Adrenocortical tumors have a distinct long non-coding RNA expression profile and LINC00271 is downregulated in malignancy

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Results: Unsupervised hierarchical clustering showed distinct clustering of ACC samples compared with NAC and ACA samples by lncRNA expression profiles. A total of 874 lncRNAs were differentially expressed between ACC and NAC. LINC00271 expression level was associated with prognosis, patients with low LINC00271 expression survived shorter than patients with high LINC00271 expression. Low LINC00271 expression was positively associated with WNT signaling, cell cycle, and chromosome segregation pathways.
Conclusions: ACC has a distinct lncRNA expression profile. LINC00271 is downregulated in ACC and is involved in biological pathways commonly dysregulated in ACC.

March 28th, 2019

Kevin E. Behrns, MD and Michael G. Sarr, MD
Editors-in-Chief
Surgery

Dear Editors,

We would like to thank the editors and reviewers again for their time reviewing the manuscript and their helpful comments. We have addressed the comments and believe the manuscript should now meet all requirements set out by the reviewers. Changes are highlighted using track changes in the revised manuscript.

We look forward to hearing your decision.

Sincerely,

Floryne O. Buishand, DVM, PhD, MRCVS

***Revision Note**

AUTHORS' RESPONSE TO REVIEWER'S COMMENTS

We sincerely appreciate the reviewers providing constructive comments. We have made changes that are highlighted in yellow in the manuscript and have addressed all issues raised by the reviewers. Below are the specific responses (in **bold** type) to the Reviewers' comments.

Reviewer 2
The authors have substantially corrected the manuscript and addressed adequately all the prior reviewer concerns. The paper is much improved and acceptable for publication.

We are delighted to read that you believe that our manuscript is acceptable for publication. Once more we would like to thank you for your thoughtful review and useful comments.

Reviewer 3
Improved manuscript after deleting claims that LINC00271 is prognostic in the title and body on manuscript. I would still ask that in the abstract results, the authors delete the word "significantly" (results line 5) which implies statistical validity. Based on the scatter gram plot of survival vs. expression with an r value of 0.5; I think the issue is not proven. I am not convinced of an association of survival. I would defer to statistical review by editorial staff.

Once more thank you for you time to review our manuscript. As per your suggestion we have deleted the word "significantly" in the abstract results.

Recorder Notes
Thank you for making substantial improvements in the manuscript. Please make the revisions requested by reviewer #3. Final acceptance by senior editors is not assured until statistical review as requested.

We have made the revision requested by reviewer #3 as we have deleted the word "significantly" from the abstract results.

Adrenocortical tumors have a distinct long non-coding RNA expression profile and LINC00271 is downregulated in malignancy*

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Abstract

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Results: Unsupervised hierarchical clustering showed distinct clustering of ACC samples compared with NAC and ACA samples by lncRNA expression profiles. A total of 874 lncRNAs were differentially expressed between ACC and NAC. *LINC00271* expression level was associated with prognosis, patients with low *LINC00271* expression survived ~~a significantly~~ shorter ~~time~~ than patients with high *LINC00271* expression. Low *LINC00271* expression was positively associated with WNT signaling, cell cycle, and chromosome segregation pathways.

Conclusions: ACC has a distinct lncRNA expression profile. *LINC00271* is downregulated in ACC and is involved in biological pathways commonly dysregulated in ACC.

Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy with an annual incidence of 0.7–2.0 cases per million people and a five-year overall survival rate ranging from 32% to 47%).[1,2] Furthermore, even after complete tumor resection, over half of the patients develop recurrent disease.[3] Patients with locally advanced and metastatic ACC often undergo therapy, which consists of a regimen, including adrenolytic mitotane plus combination chemotherapy with etoposide, doxorubicin, and cisplatin. Unfortunately, this regimen has very limited therapeutic benefit.[4] The role of adjuvant therapy for ACC is controversial because of questionable therapeutic benefit of current agents and the heterogenous prognosis.[3] Understanding the mechanism behind ACC initiation and progression could help in identifying diagnostic and prognostic markers, and therapeutic targets.

Several genomic studies of ACC have reported on distinct ACC genome-wide gene expression, micro-RNA expression, methylation and copy number alteration profiles compared with adrenal cortical adenomas (ACAs) and normal adrenal cortex (NAC).[5-10] These studies have led to the molecular classification of ACC that is relevant for predicting prognosis. Recently, long noncoding RNAs (lncRNAs) have been suggested to be dysregulated in ACC.[11] LncRNAs are RNA transcripts longer than 200 nucleotides that do not encode protein and are localized in the cell nucleus or cytoplasm.[12] The expression of lncRNAs is more tissue specific than protein-coding genes and they function as decoys, scaffolds and enhancer RNAs and are involved in chromatin remodeling, as well as transcriptional and post-transcriptional regulation.[13]

To our knowledge, the study by Glover and colleagues has been the only study that has investigated lncRNA expression profile in ACCs, ACAs and NAC.[11] They reported that the highest number of differentially expressed lncRNAs were between ACAs and NAC, with almost 3-fold less lncRNAs being differentially expressed between ACCs and NAC. This finding

suggested that changes in lncRNA expression could be an early event in the pathogenesis of both ACC and ACAs. However, this finding is in contrast to previous genome-wide analysis results that demonstrated a multistep progression in ACC, with increasing genomic changes from NAC to ACA to ACC.[8] Therefore, to further our knowledge of the role of lncRNA in ACCs, we performed lncRNA expression profiling using lncRNA microarrays to identify differentially expressed lncRNAs in ACCs compared with NACs and ACAs. We also investigated whether lncRNA expression levels were associated with ACC overall survival times.

Materials and methods

Tissue samples

Patients' tumor tissues were procured after informed consent for genetic studies on an Institutional Review Board-approved procurement clinical protocol (NCT01005654 and NCT01348698). The tissues were immediately snap frozen in liquid nitrogen and stored at -80°C. For this study, we used 11 ACA samples and nine ACC samples. Five normal NACs were obtained at the time of organ donation harvesting. These 25 tissue samples were used for lncRNA microarray profiling. In addition to these samples, an additional 10 ACC samples were included in the quantitative RT-PCR (qRT-PCR) validation (Table 1). Tumors were classified as benign when the Weiss criteria scores were less than 3 (all the benign samples included had a Weiss score of 0), and tumors were classified as ACC when the Weiss criteria scores were more than or equal to 3.[14] Only samples with at least 80% tumor cells were included for analysis.

RNA extraction

Total RNA was extracted from fresh frozen tissue samples using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality was

assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Englewood, CO, USA). Only samples with a minimum RNA integrity number of seven were included for analysis.

Microarray profiling

The ArrayStar Human LncRNA/mRNA Expression Microarray Version 3.0 (ArrayStar, Inc., Rockville, MD, USA) was used, which includes 30,586 lncRNA probes and 26,109 coding transcripts, for lncRNA profiling. RNA labeling, microarray hybridization, slide washing and scanning were performed based on the standard protocols of ArrayStar. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. The microarray specifications and derived data are accessible through National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession number GSE124531.

TaqMan real-time quantitative PCR

RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan qRT-PCR was performed using the 7900HT fast real-time PCR systems (Applied Biosystems). The reaction contained cDNA, TaqMan 2×universal PCR master mix and TaqMan gene expression assays primers (Applied Biosystems). LncRNAs were selected for validation based on three criteria: 1) availability of validated TaqMan gene expression primer/probe assays, 2) possible role in cancer, and 3) magnitude of differential expression. The gene expression assays used were: *HOTTIP* (Hs03649396_m1), *CHL1* (Hs04332026_m1), *HOXA11-AS1* (Hs_03454334_g1), *CRNDE* (HS04404483_m1), *LINC00271* (Hs03657384_m1), *FAM211A-AS1* (Hs03678558_g1), *TBXAS1* (Hs01096058_s1) and *GAPDH* (Hs99999905_m1).

Comparative genomic hybridization (CGH) array analysis

We used our previously published genome-wide CGH array data in a cohort of NAC, ACA, and ACC.[8] The *LINC100271* site was manually scanned for its copy number status using Nexus software.

Statistical and data analysis

LncRNA expression profiles of ACC samples were compared with NAC and ACA samples. The Gaussian linear model was used to calculate *P*-values, and false discovery rates (FDRs) were calculated using the Benjamini-Hochberg method for each lncRNA. LncRNAs with log2 fold change ≥2 and FDR <0.05 were defined as differentially expressed lncRNAs. Differentially expressed lncRNAs were mapped to their associated gene names and then gene set enrichment analysis (GSEA) was performed on these genes. An in-house R package, OmicPath (v 0.1) was used to perform GSEA to discover potential KEGG pathway associations for each set of differentially expressed lncRNAs. Pathways with a *P*-value < 0.05 were considered significant. Survival curves were plotted using the Kaplan-Meier methods, and differences in survival rates were determined using the log-rank test. These statistical analyses were done with GraphPad Software and $P < 0.05$ was considered significant.

The ACC cohort from the Cancer Genome Atlas (TCGA) project database (https://tcga-data.nci.nih.gov/tcga/) which included 79 patients with *HOTTIP*, *CHL1*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1* expression data, as well as follow-up information, were used to study the prognostic significance of lncRNAs. For overall survival analysis two groups were defined based on the lncRNA expression levels in the primary tumor. Those with a lncRNA

level ranked in the top half were classified into the high expression group and the rest into the low expression group based on the median value.

The gene expression profiles of ACC samples deposited in the TCGA project database were analyzed to compare expression patterns in tumors with high (n = 39) vs. low *LINC100271* expression (n = 40). The downloaded data consisted of quantified gene expression data, that were further processed using the DESeq2 package.[15] The differentialy expressed genes were annotated, and GSEA analysis was performed using the clusterProfiler package.[16]

Results

Differentially expressed lncRNAs in ACC versus NAC

Unsupervised hierarchical and heat map clustering showed distinct clustering of ACC samples compared with NAC and ACA samples (Fig. 1). Eight hundred and seventy-four lncRNAs were differentially expressed in ACC compared with NAC, of which 409 were upregulated and 465 were downregulated. The 874 differentially expressed lncRNAs corresponded to 330 annotated lncRNA genes. Among the upregulated lncRNAs, the highest log2 fold change was 8.5 for an unannotated lncRNA gene, and *RAD50* was the highest upregulated annotated lncRNA gene with a log2 fold change of 6.1. Among the downregulated lncRNAs, the highest log2 fold change was 8.3 for an unannotated lncRNA gene and 6.4 for *HAND2*, the highest downregulated annotated lncRNA gene. One hundred and eighty-three differently expressed lncRNAs had established functions in cancer development and cancer progression. Selected carcinogenesis-related lncRNAs are summarized in Table 2.

To test the validity of the microarray findings, seven lncRNAs (*HOTTIP*, *CHL1*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1*) were selected among the carcinogenesis-related differentially expressed lncRNAs and their expression was analyzed by

TaqMan qRT-PCR. The validation cohort included 19 ACC samples and 5 NAC samples. *HOTTIP*, *HOXA11-AS1* and *CRNDE* were overexpressed in ACC ($P < 0.05$) and confirmed by TaqMan qRT-PCR in the validation cohort ($P < 0.05$; Fig. 2). *LINC00271*, *FAM211A-AS1* and *TBXAS1* expression was downregulated in ACC ($P < 0.05$) and also by TaqMan qRT-PCR ($P < 0.05$) (Fig 2). The microarray result for *CHL1* was not confirmed in the validation cohort. Upregulated expression of *CHL1* was identified in the microarray analysis ($P < 0.05$) while *CHL1* was found to be not significantly upregulated by TaqMan qRT-PCR in the validation cohort.

Differentially expressed lncRNAs in ACC versus ACA

One thousand seventy-six lncRNAs were differentially expressed in ACC compared with ACA, of which 780 were upregulated and 296 were downregulated. The 1,076 differentially expressed lncRNAs corresponded to 376 annotated lncRNA genes. Among the upregulated lncRNAs, the highest log2 fold change was 8.2 for an unannotated lncRNA and 7.0 for *NKAIN4*, the highest upregulated annotated lncRNA. Among the downregulated lncRNAs, the highest log2 fold change was 7.1 for an unannotated lncRNA gene and 6.9 for *SSTR5*, the highest downregulated annotated lncRNA.

There was overlap in 206 lncRNAs as they were downregulated in ACC compared to NAC and ACC compared to ACA, and 355 lncRNAs overlapped as they were upregulated in ACC compared to NAC and ACA (Fig. 3).

Differentially expressed lncRNAs in ACA versus NAC

Unsupervised hierarchical and heat map clustering showed that NAC samples clustered together with ACA samples (Fig. 1). Only ten lncRNAs were differentially expressed in ACA compared with NAC.

Functional pathway analysis

KEGG pathway analysis of the differentially expressed and annotated lncRNAs in ACC compared with NAC and ACC compared with ACA was performed to understand the biological relevance of these lncRNAs. Twenty-one pathways were significantly enriched in ACC versus ACA and 29 pathways were significantly enriched in ACC versus NAC (Tables 3 and 4). Twelve of the altered 21 pathways were common to the comparison of ACC versus ACA and ACC versus NAC. The KEGG pathways common to both comparisons included 'Transcriptional misregulation in cancer' and 'ECM-receptor interaction'.

Prognostic lncRNAs in ACC

Using the survival data of the ACC TCGA cohort, the prognostic significance of *HOTTIP*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1* was analyzed. Only *LINC00271* expression (Fig. 4A) was found to be associated with prognosis. *LINC00271* expression levels was positively associated with survival time (Fig. 4B). Median survival time for the low-*LINC00271* expression group (n = 40) was 4.9 years, whereas it was not reached for the high-*LINC00271* expression group (n = 39) ($P < 0.019$) (Fig 4C). Student's t-tests demonstrated that *LINC00271* expression levels of stage I tumors were significantly higher than those of stage IV tumors ($P < 0.006$).

Identification of LINC00271-associated biological pathways by Gene Set Enrichment Analysis

To identify *LINC00271*-associated biological pathways, GSEA was performed using high throughput RNA-sequencing data from the TCGA ACC cohort. Among the GO gene sets, WNT signaling pathway, cell cycle, chromosome segregation and tissue morphogenesis were found to be significantly associated with low *LINC00271* expression in the ACC TCGA cohort (Fig. 5), suggesting that *LINC00271* may be involved in ACC development and/or progression through the above cancer-associated signaling pathways.

LINC00271 copy number alterations

An analysis of the *LINC00271* chromosomal locus, 6q23.3, using genome-wide CGH array data that were previously generated in a cohort of NAC, ACA, and ACC[10], was performed to examine whether the *LINC00271* site demonstrated any copy number alterations to explain its downregulated expression in ACC. One out of 11 NAC samples demonstrated a deletion at 6q23.3, whereas 2 of 18 ACA samples demonstrated deletions at 6q23.3 and two other ACA samples demonstrated amplifications at 6q23.3. The *LINC00271* locus was most unstable in ACCs, with 4 of 19 ACC samples demonstrating deletions and 4 of 19 ACC samples demonstrating amplifications of 6q23.3.

Discussion

This study demonstrated that NAC, ACA and ACC have distinct lncRNA expression profiles, and that *LINC00271*, involved in biological pathways commonly dysregulated in ACC, is a prognostic marker in ACC.

Eight hundred and seventy-four lncRNAs were differentially expressed in ACC compared with NAC, 1076 lncRNAs were differentially expressed in ACA compared with ACC, and only ten lncRNAs were differentially expressed in ACA vs. NAC. Previously, Glover and colleagues

demonstrated that the highest number of differentially expressed lncRNAs in their study were between ACA and NAC (2655 lncRNAs), while 956 lncRNAs were differentially expressed between ACC and NAC, and 85 lncRNAs were differentially expressed between ACC and ACA.[11] They suggested that changes in lncRNA expression could be an early part in the pathogenesis of both ACC and ACAs. However, our results are not entirely consistent with their findings as we found only ten lncRNAs that were differentially expressed between ACA and NAC. However, this finding is in line with the multistep hypothesis in tumorigenesis that is present in most human cancers - progressive genetic/genomic alterations increasing/accumulating from NAC to ACA to ACC as previously described in our integrated genome-wide gene expression, gene methylation, microRNA expression and CGH analysis in human NAC, ACA and ACC samples.[8] The multistep progression from NAC to ACA to ACC is further supported by our finding of 296 lncRNAs differentially expressed between ACA versus ACC and 465 lncRNAs differentially expressed between ACC vs. NAC. Overall, we found less differently expressed lncRNAs in adrenocortical tumors compared to the Glover et al. study[11] but we used a more stringent fold change cut-off to identify differentially expressed lncRNAs and the NAC samples used in our study were not adjacent normal tissue to ACAs.

In the current study, the TCGA ACC dataset was used to screen for prognostic significance of differentially expressed lncRNAs. *LINC00271* was found to be associated with malignancy, with patients with low *LINC00271* expression levels surviving a significantly shorter time than patients with high *LINC00271* expression levels. Previously, significantly lower expression of *LINC00271* has been described in invasive breast carcinoma, lung adenocarcinoma, kidney renal papillary cell carcinoma, head and neck squamous cell carcinoma and papillary thyroid cancer.[17] In addition, *LINC00271* has been found to be an independent risk factor for extrathyroidal extension, lymph node metastasis, advanced tumor stage III/IV and recurrence in

papillary thyroid cancer.[17] GSEA revealed that genes associated with cell adhesion molecules, TP53 signaling pathway, JAK/STAT signaling pathway and cell cycle were significantly enriched in papillary thyroid cancer with a low *LINC00271* expression versus papillary thyroid cancer with higher *LINC00271* expression. We also found that genes associated with cell cycle were associated with low *LINC00271* expression in the TCGA ACC cohort. Further *LINC00271* expression was positively associated with WNT signaling pathway and chromosome segregation, biological pathways commonly dysregulated in ACC.[18,19] Thus, our findings and other investigators studies suggest that *LINC00271* could contribute to abnormal activation of these pathways in a tumor suppressor manner, however further mechanistic studies are needed to test this hypothesis.

Studies have suggested that genes with causal roles in tumorigenesis are often located in chromosomal areas with copy number alterations.[20,21] Gene expression levels are directly dependent on chromosomal aneuploidies in carcinomas.[22] The strongest correlations have been found between genomic copy number and average chromosome-wide expression levels, but the expression of individual genes has also been associated with genomic copy numbers.[23] LncRNAs expression levels have been positively correlated with copy number alterations as well.[24,25] Therefore, we investigated whether copy number alterations were present at the *LINC00271* chromosomal locus, 6q23.3. This region had the highest alteration in ACC samples with 21% of samples demonstrating amplifications and another 21% demonstrating deletions, while only 11% of ACA samples had amplifications and another 11% deletions of 6q23.3. The instability of 6q23.3 might explain the dysregulated expression of *LINC00271* in ACC.

In conclusion, ACC has a distinct lncRNA expression profile, and *LINC00271* downregulation is associated with malignancy and is involved in biological pathways commonly dysregulated in ACC.

Disclosure of interest

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Tables

Table 1. Clinical features of ACA and ACC patients

	ACA*	ACC† included in microarray	ACC in validation cohort
Number of patients	11	9	10
Age (average ± SD)	46.0 years ± 18.7	52.2 years ± 14.7	46.7 years ± 13.7
Sex (female/male)	9/2	7/2	6/4
Tumor size (average ± SD)	3.8 cm ± 1.8	6.7 cm ± 5.9	5.4 cm ± 2.2
Functional	55%	44%	30%
Syndrome‡			
Adrenal hypercortisolism	3	4	3
Primary hyperaldosteronism	3	1	0
Nonfunctioning	6	4	7

* *ACA*, adrenocortical adenoma
† *ACC*, adrenocortical carcinoma
‡ Functional status at initial presentation

Table 2. Selected carcinogenesis-related differentially expressed lncRNAs between ACC and NAC

Sequence name	Gene symbol	Regulation	*P*-value	Log2 fold change	Chromosome	Relationship
ENST00000534886	*SRRM4*	Up	0.001	5.14	Chr12	Intron sense-overlapping
ENST00000472494	*HOTTIP*	Up	9.11×10^{-5}	5.05	Chr7	Bidirectional
ENST00000514846	*GRK6*	Up	9.92×10^{-6}	4.75	Chr5	Natural antisense
NR_002795	*HOXA11*	Up	4.61×10^{-5}	4.05	Chr7	Bidirectional
NR_045572	*CHL1*	Up	3.45×10^{-4}	4.16	Chr3	Exon sense-overlapping
ENST00000558031	*CRNDE*	Up	1.30×10^{-5}	2.45	Chr16	Intergenic
ENST00000502941	*HAND2*	Down	1.52×10^{-7}	6.35	Chr4	Bidirectional
ENST00000450445	*BNC2*	Down	1.36×10^{-6}	5.01	Chr9	Intronic antisense
ENST00000417354	*DNM3*	Down	4.46×10^{-6}	3.50	Chr1	Intronic antisense

NR_029394	*TBXAS1*	Down	2.15×10^{-4}	2.51	Chr7	Exon sense-overlapping
NR_026805	*LINC00271*	Down	3.99×10^{-6}	2.50	Chr6	Bidirectional
NR_027158.1	*FAM211A-AS1*	Down	2.96×10^{-3}	2.06	Chr17	Intronic antisense

Table 3. Significantly different KEGG pathways in ACC versus ACA

Pathways	Genes	*P*-value
Pathways in cancer	*ADCY2, RALBP1, CSF2RA, DAPK1, FGF13, GSK3B, BIRC5, ITGA3, MMP9, PTGER3, SLC2A1, TGFB2, PAX8, RUNX1*	1.791e-3
Vascular smooth muscle contraction	***KCNMB2, ADCY2, KCNMA1, AVPR1A, PRKCQ, PRKG1***	**3.251e-3**
Glucagon signaling pathway	***ADCY2, PRKAG2, PGAM2, PHKA2, SLC2A1***	**5.823e-3**
Malaria	*ITGAL, TGFB2, THBS4*	7.960e-3
Transcriptional misregulation in cancer	***HOXA10, HOXA11, MMP9, PAX8, HMGA2, HIST1H3G, RUNX1***	**8.716e-3**
Insulin secretion	***KCNMB2, ADCY2, KCNMA1, SLC2A1***	**1.209e-2**
Circadian rhythm	***ADCY2, PRKG1, PTGER3***	**1.266 e-2**
Salivary secretion	***NPAS2, PRKAG2***	**1.346 e-2**
Cell cycle	*ADCY2, KCNMA1, LYZ, PRKG1*	1.455 e-2
Colorectal cancer	*E2F5, GSK3B, MAD2L1, RBL2, TGFB2*	1.522 e-2
FoxO signaling pathway	*GSK3B, BIRC5, TGFB2*	1.786 e-2
Glycolysis / Gluconeogenesis	***S1PR1, PRKAG2, RBL2, BNIP3, TGFB2***	**2.149 e-2**
Ubiquitin mediated proteolysis	*PGAM2, ADPGK, FBP2*	2.307 e-2
Adipocytokine signaling pathway	***UBE2S, UBE2D4, SIAH1, UBE2G2, ITCH***	**2.367 e-2**
Signaling pathways regulating pluripotency of stem cells	*PRKAG2, PRKCQ, SLC2A1*	2.661 e-2
Bladder cancer	*ESRRB, GSK3B, PAX6, POU5F1B, PCGF1*	2.762 e-2
Insulin resistance	***DAPK1, MMP9***	**2.841 e-2**
RNA degradation	*GSK3B, PRKAG2, PRKCQ, SLC2A1*	3.180 e-2
ECM-receptor interaction	***LSM1, EXOSC10, BTG1***	**3.605 e-2**
Hypertrophic cardiomyopathy (HCM)	***SV2C, ITGA3, THBS4***	**4.385e-2**

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Table 4. Significantly different KEGG pathways in ACC versus NAC

Pathways	Genes	*P*-value
ECM-receptor interaction	***COL6A2, SV2C, ITGA3, ITGA9, THBS2***	**5.329e-4**
Circadian rhythm	***NPAS2, PRKAG2, BHLHE40***	**5.596e-4**
Vascular smooth muscle contraction	***MRVI1, KCNMA1, AVPR1A, PRKACB, PRKCQ, PRKG1***	**7.222e-4**
Adipocytokine signaling pathway	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.759e-3**
Transcriptional misregulation in cancer	***HOXA11, MEIS1, MMP9, UTY, PAX8, HMGA2, HIST1H3G***	**1.785e-3**
Cocaine addiction	*GRIN3B, GRM3, PRKACB*	3.175e-3
Salivary secretion	***KCNMA1, LYZ, PRKACB, PRKG1***	**5.0121e-3**
Glucagon signaling pathway	***PRKAG2, PGAM2, PRKACB, SLC2A1***	**8.511e-3**
Glycolysis / Gluconeogenesis	***ADH1A, PGAM2, ADPGK***	**9.687e-3**
Insulin resistance	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.161e-2**
Nicotine addiction	*CHRNA4, GRIN3B*	1.345e-2
Proteasome	*PSMA3, PSMD7*	1.740e-2
Platelet activation	*LYN, PRKACB, PRKG1, TBXAS1*	1.817e-2
Hypertrophic cardiomyopathy (HCM)	***ITGA3, ITGA9, PRKAG2***	**1.998e-2**
Hedgehog signaling pathway	*CDON, PRKACB*	2.074e-2
Endocrine and other factor-regulated calcium reabsorption	*DNM3, PRKACB*	2.074e-2
Insulin secretion	***KCNMA1, PRKACB, SLC2A1***	**2.161e-2**
Neuroactive ligand-receptor interaction	*CHRNA4, GRIN3B, GRM3, AVPR1A, RXFP1, SSTR5, THRB*	2.227e-2
Dilated cardiomyopathy	*ITGA3, ITGA9, PRKACB*	2.602e-2
Morphine addiction	*GRK6, PDE4D, PRKACB*	2.697e-2
NF-kappa B signaling pathway	*IKBKB, LYN, PRKCQ*	2.891e-2
Circadian entrainment	*PRKACB, PRKG1, CACNA1H*	3.094e-2
Regulation of lipolysis in adipocytes	***PRKACB, PRKG1***	**3.273e-2**
Long-term depression	*LYN, PRKG1*	3.900e-2
Focal adhesion	*COL6A2, ITGA3, ITGA9, PAK3, THBS2*	4.064e-2
T cell receptor signaling pathway	*IKBKB, PAK3, PRKCQ*	4.110e-2
Longevity regulating pathway – multiple species	*PRKAG2, PRKACB*	4.584e-2
Renin secretion	*KCNMA1, PRKACB*	4.584e-2
Renal cell carcinoma	*PAK3, SLC2A1*	4.946e-2

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Figure legends

Fig 1. Unsupervised hierarchical clustering and heat map of lncRNA expression between adrenocortical carcinoma (ACC), adrenocortical adenoma (ACA) and normal adrenal cortex (NAC). Each column represents a sample and each row represents a lncRNA. High relative expression is indicated in yellow and low relative expression in red.

Fig 2. TaqMan qRT-PCR validation of lncRNA microarray analysis. Fold change in comparison of adrenocortical carcinoma versus normal adrenal cortex, $*P < 0.05$.

Fig 3. Venn diagram showing the number of overlapping up- or downregulated lncRNAs in the different comparisons. ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; NAC, normal adrenal cortex.

Fig 4. *LINC00271* expression and prognosis. *A,* Distribution of *LINC00271* expression of adrenocortical carcinoma samples from the TCGA dataset. Red points were defined as low-*LINC00271* expression group and black points were defined as high-*LINC00271* expression group. *B, LINC00271* expression is positively correlated to survival time (Pearson correlation coefficient = 0.50). *C,* Kaplan-Meier plot of overall survival in the TCGA adrenocortical carcinoma cohort is shown according to *LINC00271* expression level (low vs. high).

Fig 5. *LINC00271*-associated biological signaling pathways. Based on the TCGA dataset, GSEA showed that genes associated with WNT signaling pathway, cell cycle, chromosome segregation and tissue morphogenesis were significantly enriched in lower *LINC00271* versus higher

LINC00271 expressing adrenocortical carcinomas. FDR, false discovery rate; NES, normalized enrichment score.

Figure 1

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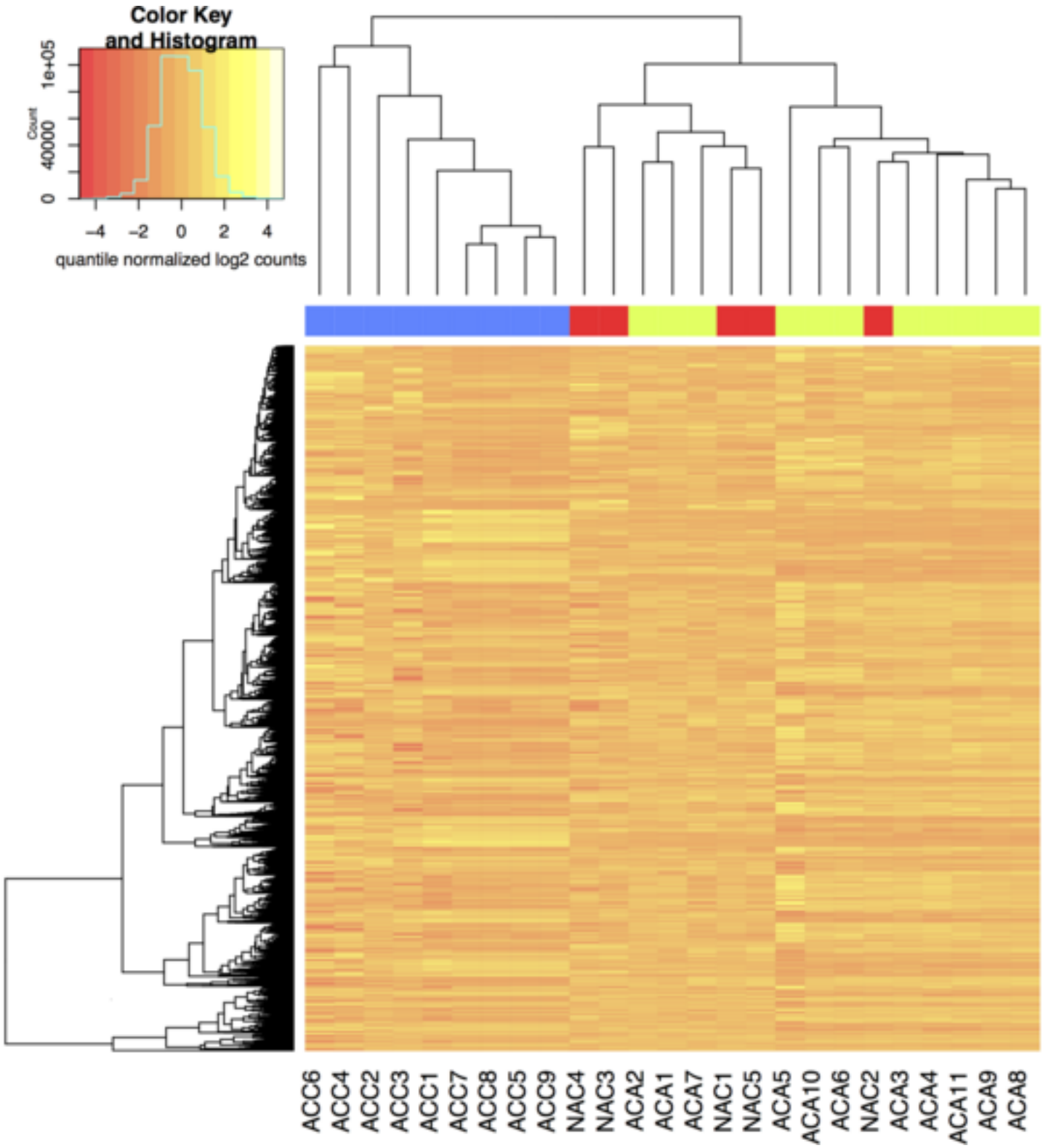

Figure 2

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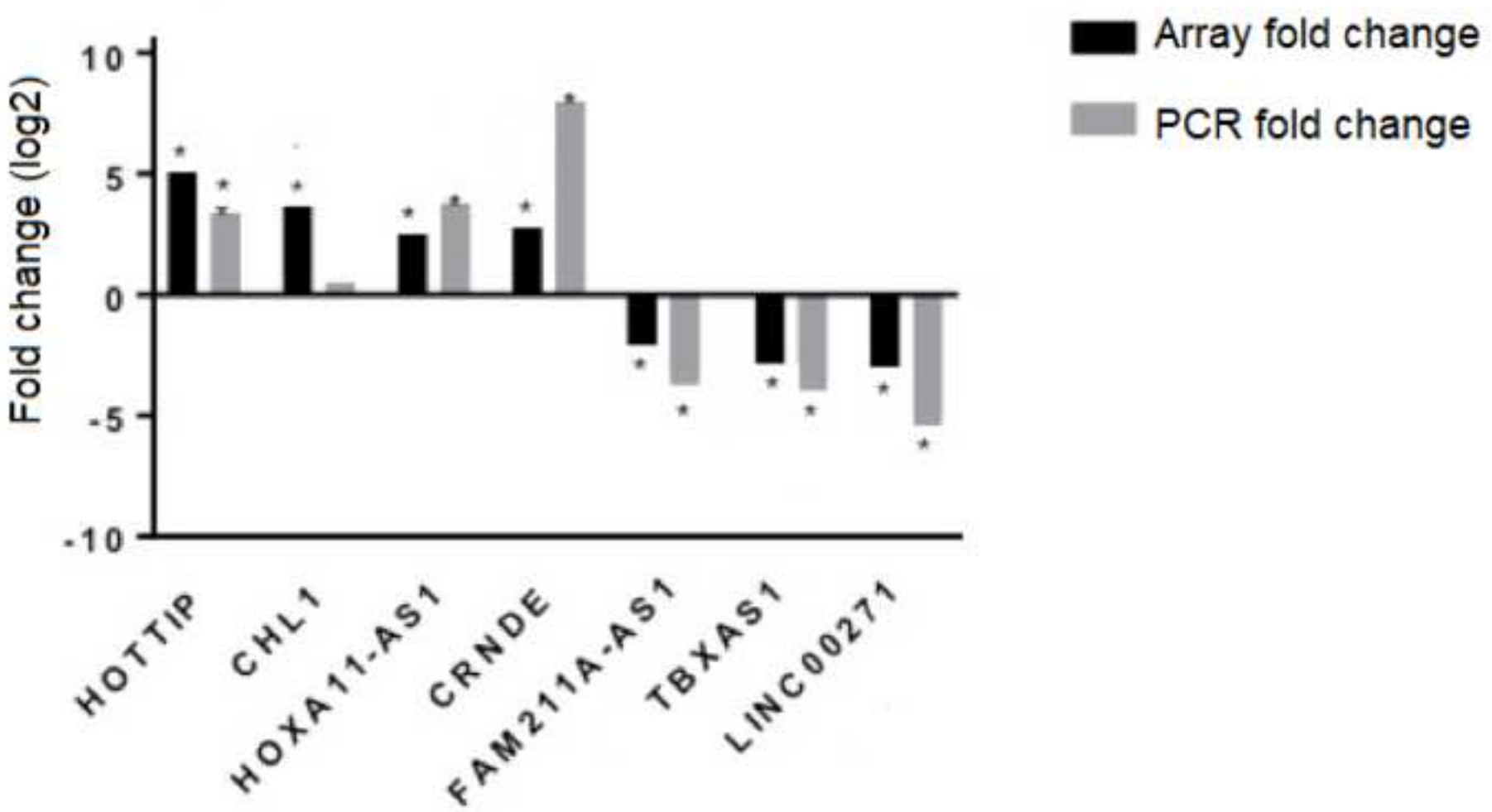

Figure 3

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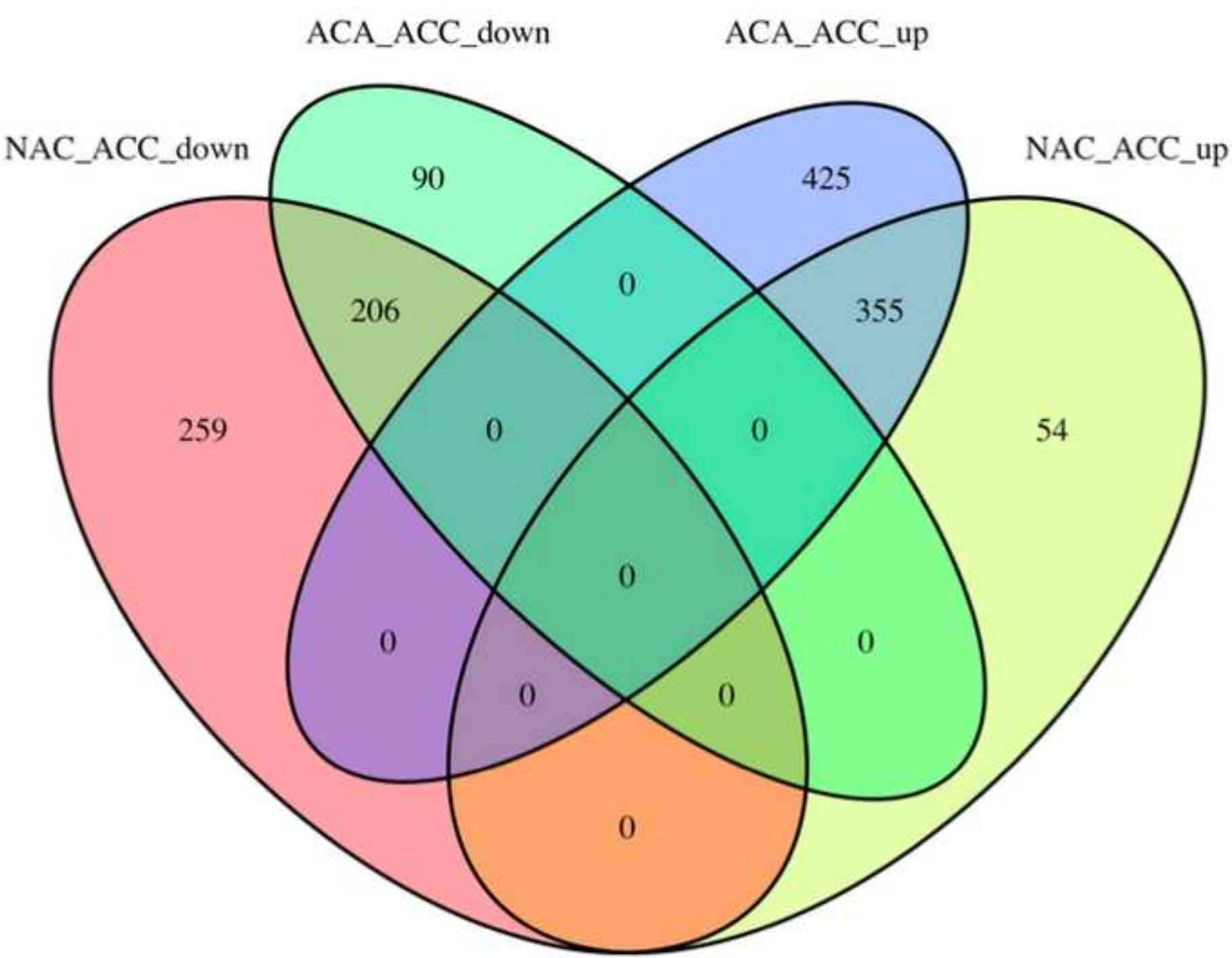

Figure 4

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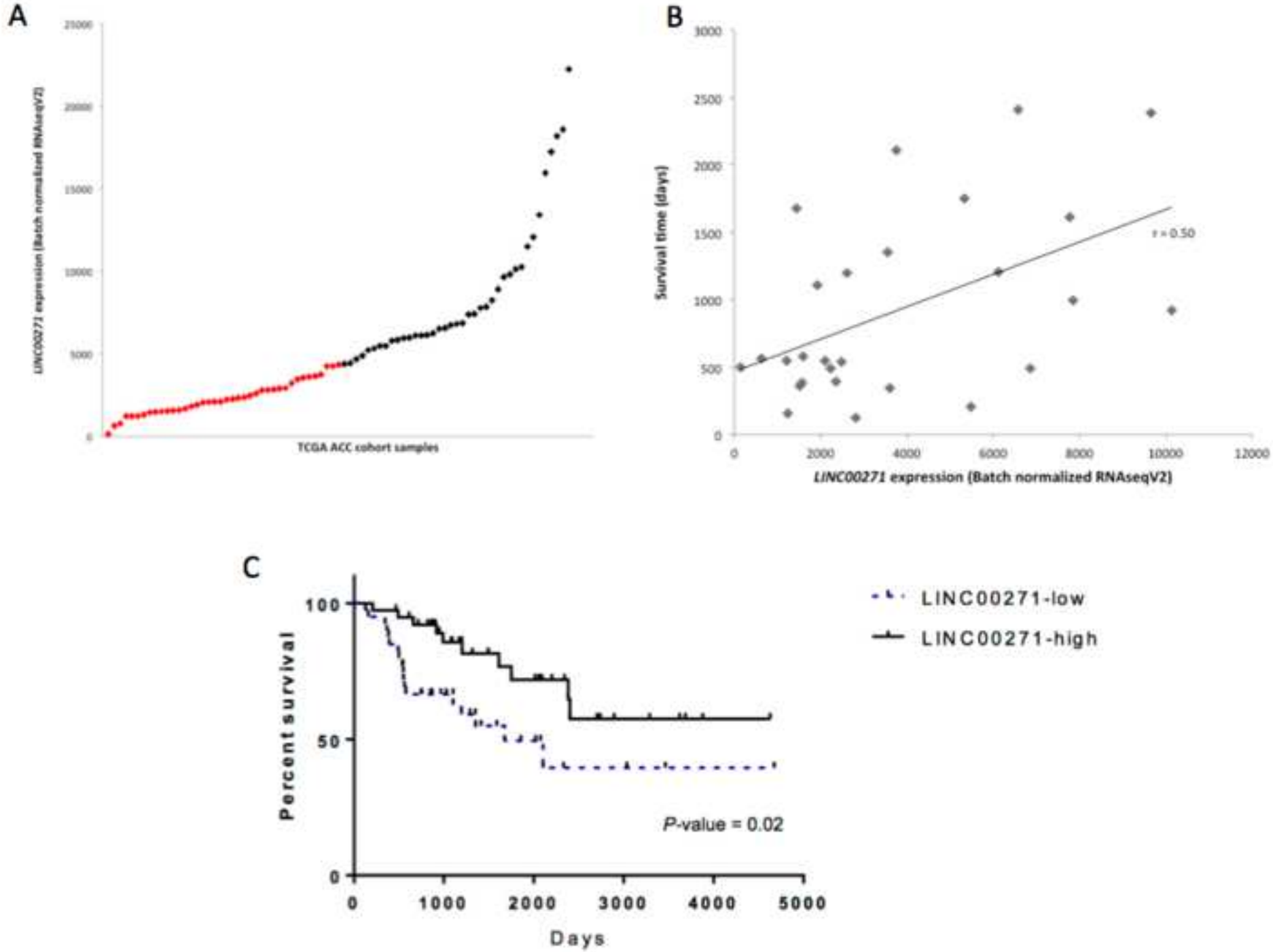

Figure 5

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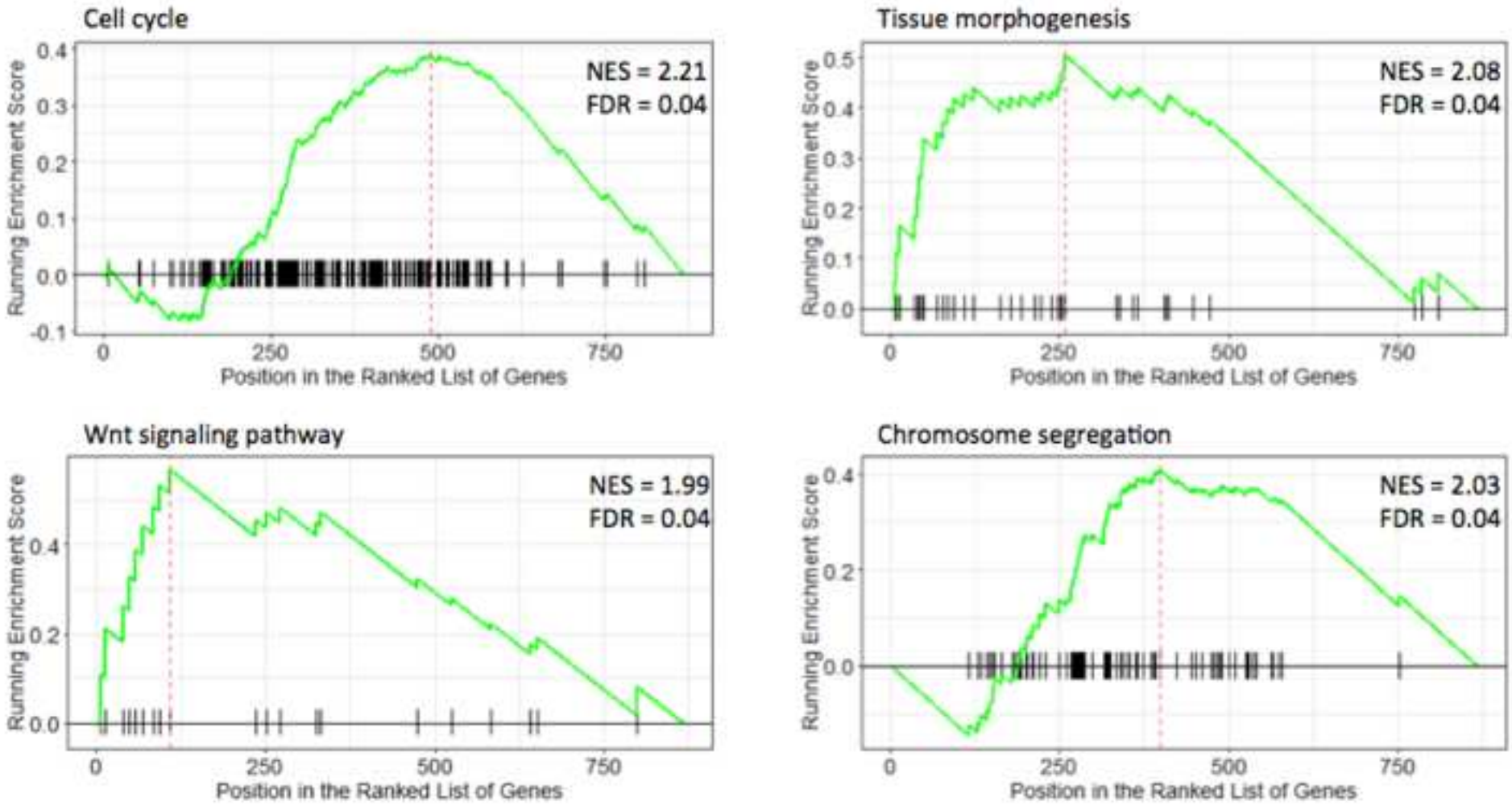

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Adrenocortical tumors have a distinct, long, non-coding RNA expression profile and LINC00271 is downregulated in malignancy*

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Abstract

Background: Adrenocortical carcinoma (ACC) is an aggressive malignancy with a low but variable overall survival rate. The role of long, noncoding RNAs (lncRNAs) in ACC is poorly understood. Thus, in this study we performed lncRNA expression profiling in ACCs, adrenocortical adenomas (ACA), and normal adrenal cortex (NAC).

Methods: LncRNA expression profile, using ArrayStar Human LncRNA/mRNA Expression Microarray V3.0, was analyzed in samples from 11 ACA, 9 ACC, and 5 NAC ~~samples~~. Differentially expressed lncRNAs were validated using TaqMan, real-time quantitative PCR with additional samples. The dataset from the ACC Cancer Genome Atlas (TCGA) project ~~dataset~~ was used to evaluate the prognostic utility of lncRNAs.

Results: Unsupervised hierarchical clustering showed distinct clustering of ACC samples compared with NAC and ACA samples by lncRNA expression profiles. A total of 874 lncRNAs were differentially expressed between ACC and NAC. *LINC00271* expression level was associated with prognosis, patients with low *LINC00271* expression survived a ~~a significantly~~ shorter time ~~time~~ than patients with high *LINC00271* expression. Low *LINC00271* expression was positively associated with WNT signaling, cell cycle, and chromosome segregation pathways.

Conclusions: ACC has a distinct lncRNA expression profile. *LINC00271* is downregulated in ACC and appears to be ~~is~~ involved in biologic~~al~~ pathways commonly dysregulated in ACC.

Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy with an annual incidence of 0.7–2.0 cases per million people and a five-year overall survival rate ranging from 32% to 47%).[1,2] Furthermore, even after complete tumor resection, over half of the patients develop recurrent disease.[3] Patients with locally advanced and metastatic ACC often undergo therapy with ~~, which consists of~~ a regimen~~,~~ including adrenolytic mitotane plus combination chemotherapy with etoposide, doxorubicin, and cisplatin. Unfortunately, this regimen has very limited therapeutic benefit.[4] The role of adjuvant therapy for ACC is controversial because of questionable therapeutic benefit of current agents and the heterogenous prognosis.[3] Understanding the mechanism behind the ~~ACC~~ initiation and progression of ACC could help in identifying diagnostic and prognostic markers, and therapeutic targets.

Several genomic studies of ACC have reported a~~on~~ distinct, ACC genome-wide gene expression and alteration profiles of~~,~~ micro-RNA expression, methylation, and copy number ~~alteration profiles~~ compared with adrenal cortical adenomas (ACAs) and normal adrenal cortex (NAC).[5-10] These studies have led to the molecular classification of ACC that is relevant for predicting prognosis. Recently, long, noncoding RNAs (lncRNAs) have been suggested to be dysregulated in ACC.[11] LncRNAs are RNA transcripts longer than 200 nucleotides that do not encode protein and are localized in the cell nucleus or cytoplasm.[12] The expression of lncRNAs is more tissue~~-~~-specific than protein-coding genes, and they function as decoys, scaffolds, and enhancer RNAs and are involved in chromatin remodeling, as well as transcriptional and post-transcriptional regulation.[13]

To our knowledge, the study by Glover and colleagues has been the only study that has investigated lncRNA expression profile in ACCs, ACAs, and NAC.[11] These investigators~~y~~ reported that the great~~high~~est number of differentially expressed lncRNAs were between ACAs

and NAC, with almost 3-fold less lncRNAs being differentially expressed between ACCs and NAC. This finding suggested that changes in lncRNA expression could be an early event in the pathogenesis of both ACC and ACAs. ~~However, t~~This finding, however, is in contrast to the results of previous, genome-wide analyses~~is results~~ that demonstrated a multistep progression in ACC, with increasing genomic changes from NAC to ACA to ACC.[8] Therefore, to further our knowledge of the role of lncRNA in ACCs, we performed lncRNA expression profiling using lncRNA microarrays to identify differentially expressed lncRNAs in ACCs compared with NACs and ACAs. We also investigated whether lncRNA expression levels were associated with ~~ACC~~ overall survival times~~.~~ of ACCs.

Materials and methods

Tissue samples

Patient~~s'~~ tumor tissues were procured after informed consent for genetic studies on a procurement clinical protocol pproved by our ~~n~~ Institutional Review Board ~~approved procurement clinical protocol~~ (NCT01005654 and NCT01348698). The tissues were immediately snap frozen in liquid nitrogen and stored at -80°C. For this study, we used 11 ACA samples and nine ACC samples. Five normal NACs were obtained at the time of organ donation harvesting. These 25 tissue samples were used for lncRNA microarray profiling. In addition to these samples, an additional 10 ACC samples were included in the quantitative RT-PCR (qRT-PCR) validation (Table 1). Tumors were classified as benign when the Weiss criteria scores were less than 3 (all the benign samples included had a Weiss score of 0), and tumors were classified as ACC, when the Weiss criteria scores were more than or equal to 3.[14] Only samples with at least 80% tumor cells were included for analysis.

RNA extraction

Total RNA was extracted from fresh frozen tissue samples using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Englewood, CO, USA). Only samples with a minimum RNA integrity number of seven were included for analysis.

Microarray profiling

The ArrayStar Human LncRNA/mRNA Expression Microarray Version 3.0 (ArrayStar, Inc., Rockville, MD, USA) ~~was~~ used for lncRNA profiling~~, which~~ includes 30,586 lncRNA probes and 26,109 coding transcripts~~, for lncRNA profiling~~. RNA labeling, microarray hybridization, slide washing, and scanning were performed based on the standard protocols of the ArrayStar. Agilent Feature Extraction software (version 11.0.1.1) which was used to analyze acquired array images. The microarray specifications and derived data are accessible through National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession number GSE124531.

TaqMan real-time quantitative PCR

RNA was reverse transcribed using the High Capacity, cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan qRT-PCR was performed using the 7900HT, fast, real-time PCR systems (Applied Biosystems). The reaction contained cDNA, TaqMan 2×universal PCR master mix, and TaqMan gene expression assays primers (Applied Biosystems). LncRNAs were selected for validation based on three criteria: 1) availability of validated TaqMan gene expression primer/probe assays, 2) possible role in cancer, and 3) magnitude of differential expression. The gene expression assays used were: *HOTTIP*

(Hs03649396_m1), *CHL1* (Hs04332026_m1), *HOXA11-AS1* (Hs_03454334_g1), *CRNDE* (HS04404483_m1), *LINC00271* (Hs03657384_m1), *FAM211A-AS1* (Hs03678558_g1), *TBXAS1* (Hs01096058_s1) and *GAPDH* (Hs99999905_m1).

Comparative genomic hybridization (CGH) array analysis

We used our previously published, genome-wide, CGH array data in a cohort of NAC, ACA, and ACC.[8] The *LINC100271* site was scanned manually ~~scanned~~ for its copy number status using Nexus software.

Statistical and data analysis

LncRNA expression profiles of ACC samples were compared with NAC and ACA samples. The Gaussian linear model was used to calculate *P*-values, and false discovery rates (FDRs) were calculated using the Benjamini-Hochberg method for each lncRNA. LncRNAs with a log2 fold change ≥2 and an FDR <0.05 were defined as differentially expressed lncRNAs. Differentially expressed lncRNAs were mapped to their associated gene names, and then a gene set enrichment analysis (GSEA) was performed on these genes. An in-house. R package, OmicPath (v 0.1) was used to perform the GSEA to discover potential KEGG pathway associations for each set of differentially expressed lncRNAs. Pathways with a *P*-value < 0.05 were considered statistically significant. Survival curves were plotted using the Kaplan-Meier methods, and differences in survival rates were determined using the log-rank test. These statistical analyses were done with GraphPad Software with ~~and~~ $P < 0.05$ ~~was~~ considered statistically significant.

The ACC cohort from the project database of the Cancer Genome Atlas (TCGA) ~~project database~~ (https://tcga-data.nci.nih.gov/tcga/) which included 79 patients with *HOTTIP*, *CHL1*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1* expression data, as well as follow-up information, were used to study the prognostic importance~~significance~~ of lncRNAs. For the overall survival analysis, two groups were defined based on the lncRNA expression levels in the primary tumor. Those with a lncRNA level ranked in the top half were classified into the high expression group and the rest into the low expression group based on the median value.

The gene expression profiles of ACC samples deposited in the TCGA project database were analyzed to compare expression patterns in tumors with high (n = 39) vs. low *LINC100271* expression (n = 40). The downloaded data consisted of quantified gene expression data~~,~~ that were further processed using the DESeq2 package.[15] The differentialy expressed genes were annotated, and GSEA analysis was performed using the clusterProfiler package.[16]

Results

Differentially expressed lncRNAs in ACC versus NAC

Unsupervised hierarchical and heat map clustering showed distinct clustering of ACC samples compared with NAC and ACA samples (Fig. 1). In these samples, 874 ~~Eight hundred and seventy four~~ lncRNAs were differentially expressed in ACC compared with NAC, of which 409 were upregulated, and 465 were downregulated. The 874 differentially expressed lncRNAs corresponded to 330 annotated lncRNA genes. Among the upregulated lncRNAs, the great~~high~~est log2 fold change was 8.5 for an unannotated lncRNA gene, and *RAD50* was the gerat~~high~~est upregulated, annotated lncRNA gene with a log2 fold change of 6.1. Among the downregulated lncRNAs, the great~~high~~est log2 fold change was 8.3 for an unannotated lncRNA gene and 6.4 for *HAND2*, the great~~high~~est downregulated annotated lncRNA gene; of these, 183~~.~~ ~~One hundred and~~

~~eighty-three~~ differently expressed lncRNAs had established functions in cancer development and cancer progression. Selected carcinogenesis-related lncRNAs are summarized in Table 2.

To test the validity of the microarray findings, seven lncRNAs (*HOTTIP*, *CHL1*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1*) were selected among the carcinogenesis-related, differentially expressed lncRNAs, and their expression was analyzed by TaqMan qRT-PCR. The validation cohort included 19 ACC samples and 5 NAC samples. *HOTTIP*, *HOXA11-AS1* and *CRNDE* were overexpressed in ACC ($P < 0.05$) and confirmed by TaqMan qRT-PCR in the validation cohort ($P < 0.05$; Fig. 2). Expression of *LINC00271*, *FAM211A-AS1* and *TBXAS1* ~~expression~~ was downregulated in ACC ($P < 0.05$) and also by TaqMan qRT-PCR ($P < 0.05$) (Fig 2). The microarray result for *CHL1* was not confirmed in the validation cohort. Upregulated expression of *CHL1* was identified in the microarray analysis ($P < 0.05$) while *CHL1* was found not to be ~~not significantly~~ upregulated by TaqMan qRT-PCR in the validation cohort.

Differentially expressed lncRNAs in ACC versus ACA

When comparing ACC with ACA, 1076 ~~One thousand seventy-six~~ lncRNAs were differentially expressed ~~in ACC compared with ACA~~, of which 780 were upregulated, and 296 were downregulated. The 1,076, differentially expressed lncRNAs corresponded to 376 annotated lncRNA genes. Among the upregulated lncRNAs, the great~~high~~est log2 fold change was 8.2 for an unannotated lncRNA and 7.0 for *NKAIN4*, the great~~high~~est upregulated annotated lncRNA. Among the downregulated lncRNAs, the great~~high~~est log2 fold change was 7.1 for an unannotated lncRNA gene and 6.9 for *SSTR5*, the great~~high~~est downregulated annotated lncRNA.

There was overlap in 206 lncRNAs as they were downregulated in ACC compared to NAC and in ACC compared to ACA, and 355 lncRNAs overlapped as they were upregulated in ACC compared to NAC and ACA (Fig. 3).

Differentially expressed lncRNAs in ACA versus NAC

Unsupervised hierarchical and heat map clustering showed that NAC samples clustered together with ACA samples (Fig. 1). Only10 ~~te~~n lncRNAs were differentially expressed in ACA compared with NAC.

Functional pathway analysis

KEGG pathway analysis of the differentially expressed and annotated lncRNAs in ACC compared with NAC and in ACC compared with ACA was performed to understand the biologic~~al~~ relevance of these lncRNAs. Twenty-one pathways were significantly enriched in ACC versus ACA and 29 pathways were significantly enriched in ACC versus NAC (Tables 3 and 4). Twelve of the altered 21 pathways were common to the comparison of ACC versus ACA and ACC versus NAC. The KEGG pathways common to both comparisons included 'Transcriptional misregulation in cancer' and 'ECM-receptor interaction'.

Prognostic lncRNAs in ACC

Using the survival data of the ACC TCGA cohort, the prognostic significance of *HOTTIP, HOXA11-AS1, CRNDE, LINC00271, FAM211A-AS1* and *TBXAS1* was analyzed. Only *LINC00271* expression (Fig. 4A) was found to be associated with prognosis. *LINC00271* expression levels were~~as~~ positively associated with survival time (Fig. 4B). Median survival time for the low-*LINC00271* expression group (n = 40) was 4.9 years, whereas it was not reached for

the high-*LINC00271* expression group (n = 39) (P < 0.019) (Fig 4C). Student's t-tests demonstrated that *LINC00271* expression levels of stage I tumors were greatsignificantly higher than those of stage IV tumors (P < 0.006).

Identification of LINC00271-associated biological pathways by Gene Set Enrichment Analysis

To identify *LINC00271*-associated biological pathways, GSEA was performed using high throughput, RNA-sequencing data from the TCGA ACC cohort. Among the GO gene sets, the WNT signaling pathway, cell cycle, chromosome segregation, and tissue morphogenesis were found to be sitatisticallygnificantly associated with low *LINC00271* expression in the ACC TCGA cohort (Fig. 5), suggesting that *LINC00271* may be involved in ACC development and/or progression through the above cancer-associated signaling pathways.

LINC00271 copy number alterations

We performed aAn analysis of the *LINC00271* chromosomal locus, 6q23.3, using genome-wide. CGH array data that were previously generated previously in a cohort of NAC, ACA, and ACC[10], was performed to examine whether the *LINC00271* site demonstrated any copy number alterations to explain its downregulated expression in ACC. One out of 11 NAC samples demonstrated a deletion at 6q23.3, whereas 2 of 18 ACA samples demonstrated deletions at 6q23.3, and two other ACA samples demonstrated amplifications at 6q23.3. The *LINC00271* locus appeared to be the was most unstable in ACCs, with 4 of 19 ACC samples demonstrating deletions, and 4 of 19 ACC samples demonstrating amplifications of 6q23.3.

Discussion

This study demonstrated that NAC, ACA, and ACC have distinct lncRNA expression

profiles, and that *LINC00271*, 2whhich aappeared to be involved in biologic~~al~~ pathways commonly dysregulated in ACC, may be~~is~~ a prognostic marker in ACC.

When compared with NAC, 874 ~~Eight hundred and seventy four~~ lncRNAs were differentially expressed in ACC, ~~compared with NAC,~~ 1076 lncRNAs were differentially expressed in ACA compared with ACC, and only t10~~en~~ lncRNAs were differentially expressed in ACA vs. NAC. Previously, Glover and colleagues demonstrated that the great~~high~~est number of differentially expressed lncRNAs in their study wa~~sere~~ between ACA and NAC (2655 lncRNAs), while 956 lncRNAs were differentially expressed between ACC and NAC, and 85 lncRNAs were differentially expressed between ACC and ACA.[11] The data of these investiagotrs ~~y~~ suggested that changes in lncRNA expression could be an early part in the pathogenesis of both ACC and ACAs. ~~In contrast, However,~~ our results are not entirely consistent with their findings, because ~~as~~ we found only 10~~ten~~ lncRNAs that were differentially expressed between ACA and NAC.; ~~However,~~ this finding is in line with the multistep hypothesis in tumorigenesis that is present in most human cancers - progressive genetic/genomic alterations increasing/accumulating from NAC to ACA to ACC as ~~previously~~ described previously in our integrated, genome-wide gene expression, gene methylation, microRNA expression, and CGH analysis in human samples form NACs, ACAs, and ACCs ~~samples~~.[8] The multistep progression from NAC to ACA to ACC is further supported by our finding of 296 lncRNAs differentially expressed between ACA versus ACC and 465 lncRNAs differentially expressed between ACC vs. NAC. Overall, we found less differently expressed lncRNAs in adrenocortical neoplasms~~tumors~~ compared to the Glover et al. study[11,] but we used a more stringent cut-off in fold ~~-~~change ~~cut-off~~ to identify differentially expressed lncRNAs, and the NAC samples used in our study were not adjacent normal tissue to ACAs.

In the current study, the TCGA ACC dataset was used to screen for prognostic

significance of differentially expressed lncRNAs. *LINC00271* was found to be associated with malignancy;, with patients with low *LINC00271* expression levels survived ing a significantly lessershorter time than patients with high *LINC00271* expression levels. Previously, a statidtifvally significantly lessower expression of *LINC00271* has been described in invasive breast carcinoma, lung adenocarcinoma, kidney renal papillary cell carcinoma, head and neck squamous cell carcinoma, and papillary thyroid cancer.[17] In addition, *LINC00271* has been found to be an independent risk factor for extrathyroidal extension, lymph node metastasis, advanced tumor stage III/IV, and recurrence in papillary thyroid cancer.[17] GSEA revealed that genes associated with cell adhesion molecules, the TP53 signaling pathway, the JAK/STAT signaling pathway, and the cell cycle were statistically ignificantly enriched in papillary thyroid cancer with a low *LINC00271* expression versus papillary thyroid cancer with greathigher *LINC00271* expression. We also found that genes associated with cell cycle were associated with low *LINC00271* expression in the TCGA ACC cohort. Further *LINC00271* expression was positively associated with gthe WNT signaling pathway and chromosome segregation which are, biological pathways commonly dysregulated in ACC.[18,19] Thus, our findings and other investigators studies suggest that *LINC00271* could contribute to abnormal activation of these pathways in a tumor suppressor manner, however, further mechanistic studies are needed to test this hypothesis.

Studies have suggested that genes with causal roles in tumorigenesis are often located in chromosomal areas with alterations in copy number alterations.[20,21] Gene expression levels are directly dependent on chromosomal aneuploidies in carcinomas.[22] The strongest correlations have been found between genomic copy number and average, chromosome-wide expression levels, but the expression of individual genes has also been associated with genomic copy numbers.[23] LncRNAs expression levels have been positively correlated with alterations in copy number alterations as well.[24,25] Therefore, we investigated whether alterations in copy number alterations

were present at the *LINC00271* chromosomal locus~~,~~ 6q23.3. This region had the great~~high~~est alteration in ACC samples with 21% of samples demonstrating amplifications and another 21% demonstrating deletions, while only 11% of ACA samples had amplifications and another 11% deletions of 6q23.3. The instability of 6q23.3 might explain the dysregulated expression of *LINC00271* in ACC.

In conclusion, ACC has a distinct lncRNA expression profile, and *LINC00271* downregulation is appears to be associated with malignancy and may be~~is~~ involved in biologic~~al~~ pathways commonly dysregulated in ACC.

Disclosure of interest

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Tables

Table 1. Clinical features of ACA and ACC patients

	ACA*	**ACC† included in microarray**	**ACC in validation cohort**
Number of patients	11	9	10
Age (average ± SD)	46.0 years ± 198.7	52.2 years ± 154.7	46.7 years ± 143.7
Sex (female/male)	9/2	7/2	6/4
Tumor size (average ± SD)	3.8 cm ± 1.8	6.7 cm ± 5.9	5.4 cm ± 2.2
Functional	55%	44%	30%
Syndrome‡			
Adrenal hypercortisolism	3	4	3
Primary hyperaldosteronism	3	1	0
Nonfunctioning	6	4	7

* *ACA*, adrenocortical adenoma
† *ACC*, adrenocortical carcinoma
‡ Functional status at initial presentation

Table 2. Selected carcinogenesis-related differentially expressed lncRNAs between ACC and NAC

Sequence name	Gene symbol	Regulation	*P*-value	Log2 fold change	Chromosome	Relationship
ENST00000534886	*SRRM4*	Up	0.001	5.14	Chr12	Intron sense-overlapping
ENST00000472494	*HOTTIP*	Up	9.11×10^{-5}	5.05	Chr7	Bidirectional
ENST00000514846	*GRK6*	Up	9.92×10^{-6}	4.75	Chr5	Natural antisense
NR_002795	*HOXA11*	Up	4.61×10^{-5}	4.05	Chr7	Bidirectional
NR_045572	*CHL1*	Up	3.45×10^{-4}	4.16	Chr3	Exon sense-overlapping
ENST00000558031	*CRNDE*	Up	1.30×10^{-5}	2.45	Chr16	Intergenic
ENST00000502941	*HAND2*	Down	1.52×10^{-7}	6.35	Chr4	Bidirectional
ENST00000450445	*BNC2*	Down	1.36×10^{-6}	5.01	Chr9	Intronic antisense
ENST00000417354	*DNM3*	Down	4.46×10^{-6}	3.50	Chr1	Intronic antisense
NR_029394	*TBXAS1*	Down	2.15×10^{-4}	2.51	Chr7	Exon sense-overlapping
NR_026805	*LINC00271*	Down	3.99×10^{-6}	2.50	Chr6	Bidirectional
NR_027158.1	*FAM211A-AS1*	Down	2.96×10^{-3}	2.06	Chr17	Intronic antisense

Table 3. Statistically significantly different KEGG pathways in ACC versus ACA

Pathways	Genes	*P*-value
Pathways in cancer	*ADCY2, RALBP1, CSF2RA, DAPK1, FGF13, GSK3B, BIRC5, ITGA3, MMP9, PTGER3, SLC2A1, TGFB2, PAX8, RUNX1*	1.791e-3
Vascular smooth muscle contraction	***KCNMB2, ADCY2, KCNMA1, AVPR1A, PRKCQ, PRKG1***	**3.251e-3**
Glucagon signaling pathway	***ADCY2, PRKAG2, PGAM2, PHKA2, SLC2A1***	**5.823e-3**
Malaria	*ITGAL, TGFB2, THBS4*	7.960e-3
Transcriptional misregulation in cancer	***HOXA10, HOXA11, MMP9, PAX8, HMGA2, HIST1H3G, RUNX1***	**8.716e-3**
Insulin secretion	***KCNMB2, ADCY2, KCNMA1, SLC2A1***	**1.209e-2**
Circadian rhythm	***ADCY2, PRKG1, PTGER3***	**1.266 e-2**
Salivary secretion	***NPAS2, PRKAG2***	**1.346 e-2**
Cell cycle	*ADCY2, KCNMA1, LYZ, PRKG1*	1.455 e-2
Colorectal cancer	*E2F5, GSK3B, MAD2L1, RBL2, TGFB2*	1.522 e-2
FoxO signaling pathway	*GSK3B, BIRC5, TGFB2*	1.786 e-2
Glycolysis / Gluconeogenesis	***S1PR1, PRKAG2, RBL2, BNIP3, TGFB2***	**2.149 e-2**
Ubiquitin mediated proteolysis	*PGAM2, ADPGK, FBP2*	2.307 e-2
Adipocytokine signaling pathway	***UBE2S, UBE2D4, SIAH1, UBE2G2, ITCH***	**2.367 e-2**
Signaling pathways regulating pluripotency of stem cells	*PRKAG2, PRKCQ, SLC2A1*	2.661 e-2
Bladder cancer	*ESRRB, GSK3B, PAX6, POU5F1B, PCGF1*	2.762 e-2
Insulin resistance	***DAPK1, MMP9***	**2.841 e-2**
RNA degradation	*GSK3B, PRKAG2, PRKCQ, SLC2A1*	3.180 e-2
ECM-receptor interaction	***LSM1, EXOSC10, BTG1***	**3.605 e-2**
Hypertrophic cardiomyopathy (HCM)	***SV2C, ITGA3, THBS4***	**4.385e-2**

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Table 4. Statistically significantly different KEGG pathways in ACC versus NAC

Pathways	Genes	*P*-value
ECM-receptor interaction	***COL6A2, SV2C, ITGA3, ITGA9, THBS2***	**5.329e-4**
Circadian rhythm	***NPAS2, PRKAG2, BHLHE40***	**5.596e-4**
Vascular smooth muscle contraction	***MRVI1, KCNMA1, AVPR1A, PRKACB, PRKCQ, PRKG1***	**7.222e-4**
Adipocytokine signaling pathway	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.759e-3**
Transcriptional misregulation in cancer	***HOXA11, MEIS1, MMP9, UTY, PAX8, HMGA2, HIST1H3G***	**1.785e-3**
Cocaine addiction	*GRIN3B, GRM3, PRKACB*	3.175e-3
Salivary secretion	***KCNMA1, LYZ, PRKACB, PRKG1***	**5.0121e-3**
Glucagon signaling pathway	***PRKAG2, PGAM2, PRKACB, SLC2A1***	**8.511e-3**
Glycolysis / Gluconeogenesis	***ADH1A, PGAM2, ADPGK***	**9.687e-3**
Insulin resistance	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.161e-2**
Nicotine addiction	*CHRNA4, GRIN3B*	1.345e-2
Proteasome	*PSMA3, PSMD7*	1.740e-2
Platelet activation	*LYN, PRKACB, PRKG1, TBXAS1*	1.817e-2
Hypertrophic cardiomyopathy (HCM)	***ITGA3, ITGA9, PRKAG2***	**1.998e-2**
Hedgehog signaling pathway	*CDON, PRKACB*	2.074e-2
Endocrine and other factor-regulated calcium reabsorption	*DNM3, PRKACB*	2.074e-2
Insulin secretion	***KCNMA1, PRKACB, SLC2A1***	**2.161e-2**
Neuroactive ligand-receptor interaction	*CHRNA4, GRIN3B, GRM3, AVPR1A, RXFP1, SSTR5, THRB*	2.227e-2
Dilated cardiomyopathy	*ITGA3, ITGA9, PRKACB*	2.602e-2
Morphine addiction	*GRK6, PDE4D, PRKACB*	2.697e-2
NF-kappa B signaling pathway	*IKBKB, LYN, PRKCQ*	2.891e-2
Circadian entrainment	*PRKACB, PRKG1, CACNA1H*	3.094e-2
Regulation of lipolysis in adipocytes	***PRKACB, PRKG1***	**3.273e-2**
Long-term depression	*LYN, PRKG1*	3.900e-2
Focal adhesion	*COL6A2, ITGA3, ITGA9, PAK3, THBS2*	4.064e-2
T cell receptor signaling pathway	*IKBKB, PAK3, PRKCQ*	4.110e-2
Longevity regulating pathway – multiple species	*PRKAG2, PRKACB*	4.584e-2
Renin secretion	*KCNMA1, PRKACB*	4.584e-2
Renal cell carcinoma	*PAK3, SLC2A1*	4.946e-2

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Figure legends

Fig 1. Unsupervised hierarchical clustering and heat map of lncRNA expression between adrenocortical carcinoma (ACC), adrenocortical adenoma (ACA), and normal adrenal cortex (NAC). Each column represents a sample, and each row represents a lncRNA. High relative expression is indicated in yellow and low relative expression in red.

Fig 2. TaqMan qRT-PCR validation of lncRNA microarray analysis. Fold-change in comparison of ACC adrenocortical carcinoma versus NAC normal adrenal cortex, *$P < 0.05$.

Fig 3. Venn diagram showing the number of overlapping up- or downregulated lncRNAs in the different comparisons. ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; NAC, normal adrenal cortex.

Fig 4. *LINC00271* expression and prognosis. *A*, Distribution of *LINC00271* expression of ACC adrenocortical carcinoma samples from the TCGA dataset. Red points were defined as low-*LINC00271* expression group and black points were defined as high-*LINC00271* expression group. *B*, *LINC00271* expression is positively correlated to survival time (Pearson correlation coefficient = 0.50). *C*, Kaplan-Meier plot of overall survival in the TCGA the ACC adrenocortical carcinoma cohort is shown according to *LINC00271* expression level (low vs. high).

Fig 5. *LINC00271*-associated biological signaling pathways. Based on the TCGA dataset, GSEA showed that genes associated with WNT signaling pathway, cell cycle, chromosome segregation and tissue morphogenesis were statistically significantly enriched in lower *LINC00271* versus

greathigher *LINC00271* expressing adrenocortical carcinomasACCs. FDR, false discovery rate; NES, normalized enrichment score.

Adrenocortical tumors have a distinct, long, non-coding RNA expression profile and LINC00271 is downregulated in malignancy*

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Abstract

Background: Adrenocortical carcinoma (ACC) is an aggressive malignancy with a low but variable overall survival rate. The role of long, noncoding RNAs (lncRNAs) in ACC is poorly understood. Thus, in this study we performed lncRNA expression profiling in ACCs, adrenocortical adenomas (ACA), and normal adrenal cortex (NAC).

Methods: LncRNA expression profile using ArrayStar Human LncRNA/mRNA Expression Microarray V3.0 was analyzed in samples from 11 ACA, 9 ACC, and 5 NAC. Differentially expressed lncRNAs were validated using TaqMan, real-time quantitative PCR with additional samples. The dataset from the ACC Cancer Genome Atlas (TCGA) project was used to evaluate the prognostic utility of lncRNAs.

Results: Unsupervised hierarchical clustering showed distinct clustering of ACC samples compared with NAC and ACA samples by lncRNA expression profiles. A total of 874 lncRNAs were differentially expressed between ACC and NAC. *LINC00271* expression level was associated with prognosis, patients with low *LINC00271* expression survived a shorter time than patients with high *LINC00271* expression. Low *LINC00271* expression was positively associated with WNT signaling, cell cycle, and chromosome segregation pathways.

Conclusions: ACC has a distinct lncRNA expression profile. *LINC00271* is downregulated in ACC and appears to be involved in biologic pathways commonly dysregulated in ACC.

Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy with an annual incidence of 0.7–2.0 cases per million people and a five-year overall survival rate ranging from 32% to 47%).[1,2] Furthermore, even after complete tumor resection, over half of the patients develop recurrent disease.[3] Patients with locally advanced and metastatic ACC often undergo therapy with a regimen including adrenolytic mitotane plus combination chemotherapy with etoposide, doxorubicin, and cisplatin. Unfortunately, this regimen has very limited therapeutic benefit.[4] The role of adjuvant therapy for ACC is controversial because of questionable therapeutic benefit of current agents and the heterogenous prognosis.[3] Understanding the mechanism behind the initiation and progression of ACC could help in identifying diagnostic and prognostic markers, and therapeutic targets.

Several genomic studies of ACC have reported a distinct, ACC genome-wide gene expression and alteration profiles of micro-RNA expression, methylation, and copy number compared with adrenal cortical adenomas (ACAs) and normal adrenal cortex (NAC).[5-10] These studies have led to the molecular classification of ACC that is relevant for predicting prognosis. Recently, long, noncoding RNAs (lncRNAs) have been suggested to be dysregulated in ACC.[11] LncRNAs are RNA transcripts longer than 200 nucleotides that do not encode protein and are localized in the cell nucleus or cytoplasm.[12] The expression of lncRNAs is more tissue-specific than protein-coding genes, and they function as decoys, scaffolds, and enhancer RNAs and are involved in chromatin remodeling, as well as transcriptional and post-transcriptional regulation.[13]

To our knowledge, the study by Glover and colleagues has been the only study that has investigated lncRNA expression profile in ACCs, ACAs, and NAC.[11] These investigators reported that the greatest number of differentially expressed lncRNAs were between ACAs and NAC, with almost 3-fold less lncRNAs being differentially expressed between ACCs and NAC.

This finding suggested that changes in lncRNA expression could be an early event in the pathogenesis of both ACC and ACAs. This finding, however, is in contrast to the results of previous, genome-wide analyses that demonstrated a multistep progression in ACC, with increasing genomic changes from NAC to ACA to ACC.[8] Therefore, to further our knowledge of the role of lncRNA in ACCs, we performed lncRNA expression profiling using lncRNA microarrays to identify differentially expressed lncRNAs in ACCs compared with NACs and ACAs. We also investigated whether lncRNA expression levels were associated with overall survival times of ACCs.

Materials and methods

Tissue samples

Patient tumor tissues were procured after informed consent for genetic studies on a procurement clinical protocol pproved by our Institutional Review Board(NCT01005654 and NCT01348698). The tissues were immediately snap frozen in liquid nitrogen and stored at -80°C. For this study, we used 11 ACA samples and nine ACC samples. Five normal NACs were obtained at the time of organ donation harvesting. These 25 tissue samples were used for lncRNA microarray profiling. In addition to these samples, an additional 10 ACC samples were included in the quantitative RT-PCR (qRT-PCR) validation (Table 1). Tumors were classified as benign when the Weiss criteria scores were less than 3 (all the benign samples included had a Weiss score of 0), and tumors were classified as ACC, when the Weiss criteria scores were more than or equal to 3.[14] Only samples with at least 80% tumor cells were included for analysis.

RNA extraction

Total RNA was extracted from fresh frozen tissue samples using an RNeasy Mini Kit

(Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Englewood, CO, USA). Only samples with a minimum RNA integrity number of seven were included for analysis.

Microarray profiling

The ArrayStar Human LncRNA/mRNA Expression Microarray Version 3.0 (ArrayStar, Inc., Rockville, MD, USA) used for lncRNA profiling includes 30,586 lncRNA probes and 26,109 coding transcripts,. RNA labeling, microarray hybridization, slide washing, and scanning were performed based on the standard protocols of the ArrayStar. Agilent Feature Extraction software (version 11.0.1.1) which was used to analyze acquired array images. The microarray specifications and derived data are accessible through National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession number GSE124531.

TaqMan real-time quantitative PCR

RNA was reverse transcribed using the High Capacity, cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan qRT-PCR was performed using the 7900HT, fast, real-time PCR systems (Applied Biosystems). The reaction contained cDNA, TaqMan 2×universal PCR master mix, and TaqMan gene expression assays primers (Applied Biosystems). LncRNAs were selected for validation based on three criteria: 1) availability of validated TaqMan gene expression primer/probe assays, 2) possible role in cancer, and 3) magnitude of differential expression. The gene expression assays used were: *HOTTIP* (Hs03649396_m1), *CHL1* (Hs04332026_m1), *HOXA11-AS1* (Hs_03454334_g1), *CRNDE* (HS04404483_m1), *LINC00271* (Hs03657384_m1), *FAM211A-AS1* (Hs03678558_g1), *TBXAS1* (Hs01096058_s1) and *GAPDH* (Hs99999905_m1).

Comparative genomic hybridization (CGH) array analysis

We used our previously published, genome-wide, CGH array data in a cohort of NAC, ACA, and ACC.[8] The *LINC100271* site was scanned manually for its copy number status using Nexus software.

Statistical and data analysis

LncRNA expression profiles of ACC samples were compared with NAC and ACA samples. The Gaussian linear model was used to calculate *P*-values, and false discovery rates (FDRs) were calculated using the Benjamini-Hochberg method for each lncRNA. LncRNAs with a log2 fold change ≥2 and an FDR <0.05 were defined as differentially expressed lncRNAs. Differentially expressed lncRNAs were mapped to their associated gene names, and then a gene set enrichment analysis (GSEA) was performed on these genes. An in-house. R package, OmicPath (v 0.1) was used to perform the GSEA to discover potential KEGG pathway associations for each set of differentially expressed lncRNAs. Pathways with a *P*-value < 0.05 were considered statistically significant. Survival curves were plotted using the Kaplan-Meier methods, and differences in survival rates were determined using the log-rank test. These statistical analyses were done with GraphPad Software with $P < 0.05$ considered statistically significant.

The ACC cohort from the project database of the Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/) which included 79 patients with *HOTTIP, CHL1, HOXA11-AS1, CRNDE, LINC00271, FAM211A-AS1* and *TBXAS1* expression data, as well as follow-up information, were used to study the prognostic importance of lncRNAs. For the overall survival

analysis, two groups were defined based on the lncRNA expression levels in the primary tumor. Those with a lncRNA level ranked in the top half were classified into the high expression group and the rest into the low expression group based on the median value.

The gene expression profiles of ACC samples deposited in the TCGA project database were analyzed to compare expression patterns in tumors with high (n = 39) vs. low *LINC100271* expression (n = 40). The downloaded data consisted of quantified gene expression data that were further processed using the DESeq2 package.[15] The differentialy expressed genes were annotated, and GSEA analysis was performed using the clusterProfiler package.[16]

Results

Differentially expressed lncRNAs in ACC versus NAC

Unsupervised hierarchical and heat map clustering showed distinct clustering of ACC samples compared with NAC and ACA samples (Fig. 1). In these samples, 874 lncRNAs were differentially expressed in ACC compared with NAC, of which 409 were upregulated, and 465 were downregulated. The 874 differentially expressed lncRNAs corresponded to 330 annotated lncRNA genes. Among the upregulated lncRNAs, the greatest log2 fold change was 8.5 for an unannotated lncRNA gene, and *RAD50* was the geratest upregulated, annotated lncRNA gene with a log2 fold change of 6.1. Among the downregulated lncRNAs, the greatest log2 fold change was 8.3 for an unannotated lncRNA gene and 6.4 for *HAND2*, the greatest downregulated annotated lncRNA gene; of these, 183 differently expressed lncRNAs had established functions in cancer development and cancer progression. Selected carcinogenesis-related lncRNAs are summarized in Table 2.

To test the validity of the microarray findings, seven lncRNAs (*HOTTIP*, *CHL1*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1*) were selected among the

carcinogenesis-related, differentially expressed lncRNAs, and their expression was analyzed by TaqMan qRT-PCR. The validation cohort included 19 ACC samples and 5 NAC samples. *HOTTIP*, *HOXA11-AS1* and *CRNDE* were overexpressed in ACC ($P < 0.05$) and confirmed by TaqMan qRT-PCR in the validation cohort ($P < 0.05$; Fig. 2). Expression of *LINC00271*, *FAM211A-AS1* and *TBXAS1* was downregulated in ACC ($P < 0.05$) and also by TaqMan qRT-PCR ($P < 0.05$) (Fig 2). The microarray result for *CHL1* was not confirmed in the validation cohort. Upregulated expression of *CHL1* was identified in the microarray analysis ($P < 0.05$) while *CHL1* was found not to be upregulated by TaqMan qRT-PCR in the validation cohort.

Differentially expressed lncRNAs in ACC versus ACA

When comparing ACC with ACA, 1076 lncRNAs were differentially expressed , of which 780 were upregulated, and 296 were downregulated. The 1,076, differentially expressed lncRNAs corresponded to 376 annotated lncRNA genes. Among the upregulated lncRNAs, the greatest log2 fold change was 8.2 for an unannotated lncRNA and 7.0 for *NKAIN4*, the greatest upregulated annotated lncRNA. Among the downregulated lncRNAs, the greatest log2 fold change was 7.1 for an unannotated lncRNA gene and 6.9 for *SSTR5*, the greatest downregulated annotated lncRNA.

There was overlap in 206 lncRNAs as they were downregulated in ACC compared to NAC and in ACC compared to ACA, and 355 lncRNAs overlapped as they were upregulated in ACC compared to NAC and ACA (Fig. 3).

Differentially expressed lncRNAs in ACA versus NAC

Unsupervised hierarchical and heat map clustering showed that NAC samples clustered together with ACA samples (Fig. 1). Only10n lncRNAs were differentially expressed in ACA compared with NAC.

Functional pathway analysis

KEGG pathway analysis of the differentially expressed and annotated lncRNAs in ACC compared with NAC and in ACC compared with ACA was performed to understand the biologic relevance of these lncRNAs. Twenty-one pathways were significantly enriched in ACC versus ACA and 29 pathways were significantly enriched in ACC versus NAC (Tables 3 and 4). Twelve of the altered 21 pathways were common to the comparison of ACC versus ACA and ACC versus NAC. The KEGG pathways common to both comparisons included 'Transcriptional misregulation in cancer' and 'ECM-receptor interaction'.

Prognostic lncRNAs in ACC

Using the survival data of the ACC TCGA cohort, the prognostic significance of *HOTTIP*, *HOXA11-AS1*, *CRNDE*, *LINC00271*, *FAM211A-AS1* and *TBXAS1* was analyzed. Only *LINC00271* expression (Fig. 4A) was found to be associated with prognosis. *LINC00271* expression levels were positively associated with survival time (Fig. 4B). Median survival time for the low-*LINC00271* expression group (n = 40) was 4.9 years, whereas it was not reached for the high-*LINC00271* expression group (n = 39) (P < 0.019) (Fig 4C). Student's t-tests demonstrated that *LINC00271* expression levels of stage I tumors were greater than those of stage IV tumors (P < 0.006).

Identification of LINC00271-associated biologic pathways by Gene Set Enrichment Analysis

To identify *LINC00271*-associated biologic pathways, GSEA was performed using high throughput, RNA-sequencing data from the TCGA ACC cohort. Among the GO gene sets, the WNT signaling pathway, cell cycle, chromosome segregation, and tissue morphogenesis were found to be statistically associated with low *LINC00271* expression in the ACC TCGA cohort (Fig. 5), suggesting that *LINC00271* may be involved in ACC development and/or progression through the above cancer-associated signaling pathways.

LINC00271 copy number alterations

We performed an analysis of the *LINC00271* chromosomal locus 6q23.3 using genome-wide. CGH array data that were generated previously in a cohort of NAC, ACA, and ACC[10] to examine whether the *LINC00271* site demonstrated any copy number alterations to explain its downregulated expression in ACC. One of 11 NAC samples demonstrated a deletion at 6q23.3, whereas 2 of 18 ACA samples demonstrated deletions at 6q23.3, and two other ACA samples demonstrated amplifications at 6q23.3. The *LINC00271* locus appeared to be the most unstable in ACCs, with 4 of 19 ACC samples demonstrating deletions, and 4 of 19 ACC samples demonstrating amplifications of 6q23.3.

Discussion

This study demonstrated that NAC, ACA, and ACC have distinct lncRNA expression profiles, and that *LINC00271*, 2whhich aappeared to be involved in biologic pathways commonly dysregulated in ACC, may be a prognostic marker in ACC.

When compared with NAC, 874 lncRNAs were differentially expressed in ACC, 1076 lncRNAs were differentially expressed in ACA compared with ACC, and only t10 lncRNAs were differentially expressed in ACA vs. NAC. Previously, Glover and colleagues demonstrated that

the greatest number of differentially expressed lncRNAs in their study was between ACA and NAC (2655 lncRNAs), while 956 lncRNAs were differentially expressed between ACC and NAC, and 85 lncRNAs were differentially expressed between ACC and ACA.[11] The data of these investiagotrs suggested that changes in lncRNA expression could be an early part in the pathogenesis of both ACC and ACAs.In contrast, our results are not entirely consistent with their findings, because we found only 10lncRNAs that were differentially expressed between ACA and NAC.;this finding is in line with the multistep hypothesis in tumorigenesis that is present in most human cancers - progressive genetic/genomic alterations increasing/accumulating from NAC to ACA to ACC as described previously in our integrated, genome-wide gene expression, gene methylation, microRNA expression, and CGH analysis in human samples form NACs, ACAs, and ACCs.[8] The multistep progression from NAC to ACA to ACC is further supported by our finding of 296 lncRNAs differentially expressed between ACA versus ACC and 465 lncRNAs differentially expressed between ACC vs. NAC. Overall, we found less differently expressed lncRNAs in adrenocortical neoplasmscompared to the Glover et al. study[11,] but we used a more stringent cut-off in fold-change to identify differentially expressed lncRNAs, and the NAC samples used in our study were not adjacent normal tissue to ACAs.

In the current study, the TCGA ACC dataset was used to screen for prognostic significance of differentially expressed lncRNAs. *LINC00271* was found to be associated with malignancy; patients with low *LINC00271* expression levels survived a significantly lesser time than patients with high *LINC00271* expression levels. Previously, a statidtifvally lesser expression of *LINC00271* has been described in invasive breast carcinoma, lung adenocarcinoma, kidney renal papillary cell carcinoma, head and neck squamous cell carcinoma, and papillary thyroid cancer.[17] In addition, *LINC00271* has been found to be an independent risk factor for extrathyroidal extension, lymph node metastasis, advanced tumor stage III/IV, and recurrence in

papillary thyroid cancer.[17] GSEA revealed that genes associated with cell adhesion molecules, the TP53 signaling pathway, the JAK/STAT signaling pathway, and the cell cycle were statistically enriched in papillary thyroid cancer with a low *LINC00271* expression versus papillary thyroid cancer with greater *LINC00271* expression. We also found that genes associated with cell cycle were associated with low *LINC00271* expression in the TCGA ACC cohort. Further *LINC00271* expression was positively associated with gthe WNT signaling pathway and chromosome segregation which are biologic pathways commonly dysregulated in ACC.[18,19] Thus, our findings and other investigators studies suggest that *LINC00271* could contribute to abnormal activation of these pathways in a tumor suppressor manner, however, further mechanistic studies are needed to test this hypothesis.

Studies have suggested that genes with causal roles in tumorigenesis are often located in chromosomal areas with alterations in copy number.[20,21] Gene expression levels are directly dependent on chromosomal aneuploidies in carcinomas.[22] The strongest correlations have been found between genomic copy number and average, chromosome-wide expression levels, but the expression of individual genes has also been associated with genomic copy numbers.[23] LncRNAs expression levels have been positively correlated with alterations in copy number as well.[24,25] Therefore, we investigated whether alterations in copy number were present at the *LINC00271* chromosomal locus 6q23.3. This region had the greatest alteration in ACC samples with 21% of samples demonstrating amplifications and another 21% demonstrating deletions, while only 11% of ACA samples had amplifications and another 11% deletions of 6q23.3. The instability of 6q23.3 might explain the dysregulated expression of *LINC00271* in ACC.

In conclusion, ACC has a distinct lncRNA expression profile, and *LINC00271* downregulation is appears to be associated with malignancy and may be involved in biologic pathways commonly dysregulated in ACC.

COI/Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Tables

Table 1. Clinical features of ACA and ACC patients

	ACA*	**ACC† included in microarray**	**ACC in validation cohort**
Number of patients	11	9	10
Age (average ± SD)	46 years ± 19	52 years ± 15	47 years ± 14
Sex (female/male)	9/2	7/2	6/4
Tumor size (average ± SD)	3.8 cm ± 1.8	6.7 cm ± 5.9	5.4 cm ± 2.2
Functional	55%	44%	30%
Syndrome‡			
Adrenal hypercortisolism	3	4	3
Primary hyperaldosteronism	3	1	0
Nonfunctioning	6	4	7

* *ACA*, adrenocortical adenoma
† *ACC*, adrenocortical carcinoma
‡ Functional status at initial presentation

Table 2. Selected carcinogenesis-related differentially expressed lncRNAs between ACC and NAC

Sequence name	**Gene symbol**	**Regulation**	***P*-value**	**Log2 fold change**	**Chromosome**	**Relationship**
ENST00000534886	*SRRM4*	Up	0.001	5.14	Chr12	Intron sense-overlapping
ENST00000472494	*HOTTIP*	Up	9.11×10^{-5}	5.05	Chr7	Bidirectional
ENST00000514846	*GRK6*	Up	9.92×10^{-6}	4.75	Chr5	Natural antisense
NR_002795	*HOXA11*	Up	4.61×10^{-5}	4.05	Chr7	Bidirectional
NR_045572	*CHL1*	Up	3.45×10^{-4}	4.16	Chr3	Exon sense-

						overlapping
ENST00000558031	*CRNDE*	Up	1.30×10^{-5}	2.45	Chr16	Intergenic
ENST00000502941	*HAND2*	Down	1.52×10^{-7}	6.35	Chr4	Bidirectional
ENST00000450445	*BNC2*	Down	1.36×10^{-6}	5.01	Chr9	Intronic antisense
ENST00000417354	*DNM3*	Down	4.46×10^{-6}	3.50	Chr1	Intronic antisense
NR_029394	*TBXAS1*	Down	2.15×10^{-4}	2.51	Chr7	Exon sense-overlapping
NR_026805	*LINC00271*	Down	3.99×10^{-6}	2.50	Chr6	Bidirectional
NR_027158.1	*FAM211A-AS1*	Down	2.96×10^{-3}	2.06	Chr17	Intronic antisense

Table 3. Statistically significant different KEGG pathways in ACC versus ACA

Pathways	Genes	*P*-value
Pathways in cancer	*ADCY2, RALBP1, CSF2RA, DAPK1, FGF13, GSK3B, BIRC5, ITGA3, MMP9, PTGER3, SLC2A1, TGFB2, PAX8, RUNX1*	1.791e-3
Vascular smooth muscle contraction	***KCNMB2, ADCY2, KCNMA1, AVPR1A, PRKCQ, PRKG1***	**3.251e-3**
Glucagon signaling pathway	***ADCY2, PRKAG2, PGAM2, PHKA2, SLC2A1***	**5.823e-3**
Malaria	*ITGAL, TGFB2, THBS4*	7.960e-3
Transcriptional misregulation in cancer	***HOXA10, HOXA11, MMP9, PAX8, HMGA2, HIST1H3G, RUNX1***	**8.716e-3**
Insulin secretion	***KCNMB2, ADCY2, KCNMA1, SLC2A1***	**1.209e-2**
Circadian rhythm	***ADCY2, PRKG1, PTGER3***	**1.266 e-2**
Salivary secretion	***NPAS2, PRKAG2***	**1.346 e-2**
Cell cycle	*ADCY2, KCNMA1, LYZ, PRKG1*	1.455 e-2
Colorectal cancer	*E2F5, GSK3B, MAD2L1, RBL2, TGFB2*	1.522 e-2
FoxO signaling pathway	*GSK3B, BIRC5, TGFB2*	1.786 e-2
Glycolysis / Gluconeogenesis	***S1PR1, PRKAG2, RBL2, BNIP3, TGFB2***	**2.149 e-2**
Ubiquitin mediated proteolysis	*PGAM2, ADPGK, FBP2*	2.307 e-2
Adipocytokine signaling pathway	***UBE2S, UBE2D4, SIAH1, UBE2G2, ITCH***	**2.367 e-2**
Signaling pathways regulating pluripotency of stem cells	*PRKAG2, PRKCQ, SLC2A1*	2.661 e-2
Bladder cancer	*ESRRB, GSK3B, PAX6, POU5F1B, PCGF1*	2.762 e-2
Insulin resistance	***DAPK1, MMP9***	**2.841 e-2**
RNA degradation	*GSK3B, PRKAG2, PRKCQ, SLC2A1*	3.180 e-2
ECM-receptor interaction	***LSM1, EXOSC10, BTG1***	**3.605 e-2**
Hypertrophic cardiomyopathy (HCM)	***SV2C, ITGA3, THBS4***	**4.385e-2**

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Table 4. Statistically significantly different KEGG pathways in ACC versus NAC

Pathways	Genes	*P*-value
ECM-receptor interaction	***COL6A2, SV2C, ITGA3, ITGA9, THBS2***	**5.329e-4**
Circadian rhythm	***NPAS2, PRKAG2, BHLHE40***	**5.596e-4**
Vascular smooth muscle contraction	***MRVI1, KCNMA1, AVPR1A, PRKACB, PRKCQ, PRKG1***	**7.222e-4**
Adipocytokine signaling pathway	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.759e-3**
Transcriptional misregulation in cancer	***HOXA11, MEIS1, MMP9, UTY, PAX8, HMGA2, HIST1H3G***	**1.785e-3**
Cocaine addiction	*GRIN3B, GRM3, PRKACB*	3.175e-3
Salivary secretion	***KCNMA1, LYZ, PRKACB, PRKG1***	**5.0121e-3**
Glucagon signaling pathway	***PRKAG2, PGAM2, PRKACB, SLC2A1***	**8.511e-3**
Glycolysis / Gluconeogenesis	***ADH1A, PGAM2, ADPGK***	**9.687e-3**
Insulin resistance	***IKBKB, PRKAG2, PRKCQ, SLC2A1***	**1.161e-2**
Nicotine addiction	*CHRNA4, GRIN3B*	1.345e-2
Proteasome	*PSMA3, PSMD7*	1.740e-2
Platelet activation	*LYN, PRKACB, PRKG1, TBXAS1*	1.817e-2
Hypertrophic cardiomyopathy (HCM)	***ITGA3, ITGA9, PRKAG2***	**1.998e-2**
Hedgehog signaling pathway	*CDON, PRKACB*	2.074e-2
Endocrine and other factor-regulated calcium reabsorption	*DNM3, PRKACB*	2.074e-2
Insulin secretion	***KCNMA1, PRKACB, SLC2A1***	**2.161e-2**
Neuroactive ligand-receptor interaction	*CHRNA4, GRIN3B, GRM3, AVPR1A, RXFP1, SSTR5, THRB*	2.227e-2
Dilated cardiomyopathy	*ITGA3, ITGA9, PRKACB*	2.602e-2
Morphine addiction	*GRK6, PDE4D, PRKACB*	2.697e-2
NF-kappa B signaling pathway	*IKBKB, LYN, PRKCQ*	2.891e-2
Circadian entrainment	*PRKACB, PRKG1, CACNA1H*	3.094e-2
Regulation of lipolysis in adipocytes	***PRKACB, PRKG1***	**3.273e-2**
Long-term depression	*LYN, PRKG1*	3.900e-2
Focal adhesion	*COL6A2, ITGA3, ITGA9, PAK3, THBS2*	4.064e-2
T cell receptor signaling pathway	*IKBKB, PAK3, PRKCQ*	4.110e-2
Longevity regulating pathway – multiple species	*PRKAG2, PRKACB*	4.584e-2
Renin secretion	*KCNMA1, PRKACB*	4.584e-2
Renal cell carcinoma	*PAK3, SLC2A1*	4.946e-2

Note Pathways common to the comparison of ACC versus ACA and ACC versus NAC are written in bold type

Figure legends

Fig 1. Unsupervised hierarchical clustering and heat map of lncRNA expression between adrenocortical carcinoma (ACC), adrenocortical adenoma (ACA), and normal adrenal cortex (NAC). Each column represents a sample, and each row represents a lncRNA. High relative expression is indicated in yellow and low relative expression in red.

Fig 2. TaqMan qRT-PCR validation of lncRNA microarray analysis. Fold-change in comparison of ACCversusNAC, $*P < 0.05$.

Fig 3. Venn diagram showing the number of overlapping up- or downregulated lncRNAs in the different comparisons.

Fig 4. *LINC00271* expression and prognosis. *A*, Distribution of *LINC00271* expression of ACC samples from the TCGA dataset. Red points were defined as low-*LINC00271* expression group and black points were defined as high-*LINC00271* expression group. *B*, *LINC00271* expression is positively correlated to survival time (Pearson correlation coefficient = 0.50). *C*, Kaplan-Meier plot of overall survival in the TCGA the ACCcohort is shown according to *LINC00271* expression level (low vs. high).

Fig 5. *LINC00271*-associated biologic signaling pathways. Based on the TCGA dataset, GSEA showed that genes associated with WNT signaling pathway, cell cycle, chromosome segregation and tissue morphogenesis were statistically enriched in lower *LINC00271* versus greater *LINC00271* expressing ACCs. FDR, false discovery rate; NES, normalized enrichment score.

Discussion of Paper Number 22

ADRENOCORTICAL TUMORS HAVE A DISTINCT LONG NON-CODING RNA EXPRESSION PROFILE AND LINC00271 IS A PROGNOSTIC MARKER IN ADRENOCORTICAL CARCINOMA

DISCUSSANT

DR. XAVIER KEUTGEN (Chicago, IL): First of all, did you look at LINC00271 expression in your cell lines?

CLOSING DISCUSSANT

DR. FLORYNE O. BUISHAND: Yes, we also looked at LINC00271 expression in cell lines, and it is expressed. We also tried to knock it down; Unfortunately, that did not work.

DISCUSSANT

DR. XAVIER KEUTGEN (Chicago, IL): That would have been my next question because that may help you find out if it truly has an impact on the Wnt pathway, but it's basically downregulated as far as you could tell. Good.

Then the second question is, what do we do with this? Should this change our diagnostic or therapeutic approach?

CLOSING DISCUSSANT

DR. FLORYNE O. BUISHAND: Obviously, the study is not powered adequately to say this is really an excellent prognostic factor. So it needs more study before we can actually incorporate it in the current treatment protocols.

DISCUSSANT

DR. MARK COHEN (Ann Arbor, MI): How confident are you that this is really a marker for malignancy given that only 4 out of 19 of your cancers showed alterations in the long non-coding RNA and a certain percentage of adenomas do as well.

CLOSING DISCUSSANT

DR. FLORYNE O. BUISHAND: Obviously, we only had to look at the copy number status to see if we could find an explanation for the dysregulated expression. Those numbers are low, so we cannot be certain that it is really due to dysregulated copy number status. But I do think that we have shown with this work that LINC00271 expression is correlated and associated with malignancy and survival.

DISCUSSANT

DR. EMAD KANDIL (New Orleans, LA): The first question is about the design of the study. You decided to do the microRNA-seq on your specimens and then went back to the TCGA database. Usually, you do the bioinformatic analysis in the TCGA database, identify a panel, and then go back to your specimens and try to find this. I wonder why you decided to do it the other way around.

You had the seven genes or seven LINC RNAs, and then you decided to just focus on the LINC00271. I wonder how that happened. Why not a panel? Specifically, I think if you are looking at prognosis, the panel would be more informative than just trying to focus on one.

CLOSING DISCUSSANT

DR. FLORYNE O. BUISHAND: To address your first question, I think we could have gone back from the high throughput analysis that we did with the microarray. We started with that, and

then went on to have a look at the TCGA database, and we have could have gone back to our own samples. But, unfortunately, I don't think that we have enough samples to actually make stronger conclusions than using the TCGA data set.

Regarding the second part of your question, I think I forgot to mention that we did have a look at all those six validated LINC RNAs, whether they had prognostic significance, and we only found prognostic significance for LINC00271. So the other five did not have any prognostic significance.

DISCUSSANT

DR. MICHAEL DEMEURE (Newport Beach, CA): TCGA data are actually slanted toward early resectable lesions. Those are the operative samples, for the most part. So as you added it to your multi-step progression, was there a difference between localized and metastatic

adrenal cancers in terms of the long non-coding RNA?

I'm interested in if you are hypothesizing that you are seeing progression from adenoma to carcinoma. Given the TCGA is really slanted toward resected operative samples, and thus by definition earlier stage tumors, do you have a cohort of stage 4 disease? And could you link continuation of that progression with the long non-coding RNA?

CLOSING DISCUSSANT

DR. FLORYNE O. BUISHAND: Thank you so much for the excellent suggestion. Basically, we concluded on our limited sample set that there is a stepwise progression, and it would be really good to follow up on that finding. But you are correct, we cannot really elaborate on that, given the fact that the TCGA database only has resectable samples.

TOC Statement

Click here to view linked References

TOC Statement- 19-aaes-22

Adrenocortical carcinoma (ACC) has a distinct lncRNA expression profile and LINC00271 is a prognostic marker in ACC. The importance of this finding is that LINC00271 may serve as a potential predictor for poor clinical outcomes.